XXVIII. THE SECONDARY OXIDATION OF SOME SUBSTANCES OF PHYSIOLOGICAL INTEREST.

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In a recent paper by Thurlow [1925] evidence was given for the formation of hydrogen peroxide during certain enzymic and non-enzymic oxidations and it was shown that in the presence of peroxidase this hydrogen peroxide could bring about the oxidation of nitrites. Many important oxidations of the cell may possibly be brought about by means of a similar mechanism and the present paper describes work carried out to study the effect of replacing nitrite by other substances of physiological importance.

In various experiments with the xanthine oxidase of milk, we had observed that in certain cases, when milk peroxidase was added to the xanthine oxidase with hypoxanthine, the total oxygen uptake of the system was considerably in excess of that theoretically required for the oxidation of the hypoxanthine to uric acid. This observation suggested that some substance other than hypoxanthine might be undergoing oxidation, possibly in a manner comparable to the oxidation of nitrite in the systems mentioned above. The xanthine oxidase preparations had been made according to the method described by Dixon and Thurlow [1924], involving precipitation of the enzyme together with the caseinogen from milk by half saturation with ammonium sulphate. Part of the fat had been subsequently removed by extraction of the partially dried precipitate with ether. It was thought that the substance undergoing oxidation might be fat which had escaped removal during the ether extraction. In order to investigate this possibility experiments were carried out to compare the oxygen uptake of a system containing unextracted enzyme preparation with one containing the same enzyme preparation from which fat had been removed by prolonged extraction with ether.

A xanthine oxidase preparation was made by half saturating milk with ammonium sulphate. The precipitate, after being dried on filter paper, was shaken once with twice its own volume of ether. This process results in a removal of most of the colouring matter of milk but leaves behind a large quantity of the fat. The precipitate was then dried *in vacuo* and powdered. This will be referred to as the "unextracted preparation."

In order to obtain a fat-free preparation, a portion of this was shaken again with twice its volume of ether, dried *in vacuo* and finely powdered. This second extraction with ether facilitates the grinding of the dried product to a

fine powder. The powder was then extracted continuously with ether in a Soxhlet apparatus for about 1 hour, the excess ether absorbed with filter paper, and the product dried *in vacuo*. This will be called the "extracted preparation." A solution of the enzyme was made by adding the powdered preparation to phosphate buffer of p_H 7·6 and allowing it to stand in a water-bath at 45° for about half an hour. The resulting solution, which is acid, is brought back to p_H 7·6 by addition of the requisite amount of 10 % sodium hydroxide solution. The actual concentration of enzyme solution used varied according to the activity of the particular enzyme preparation, the usual concentration being 3 g. of the dry preparation in 10 cc. of solution.

The peroxidase preparation was made from milk according to the method described by Thurlow [1925], namely, by saturating with ammonium sulphate the filtrate which remained from the milk which had previously been half saturated with ammonium sulphate. The peroxidase is brought down on the lactalbumin and this precipitate was filtered off and dissolved in a small amount of phosphate buffer of p_H 7·6, the necessary amount of sodium hydroxide being added to restore the p_H to 7·6.

The hypoxanthine, or xanthine, used was finely powdered, suspended in water and brought into solution by addition of the minimum amount of sodium hydroxide. The usual concentration employed was 10 mg. per cc.

The oxygen uptake was measured by means of the Barcroft micro-respirometer and unless otherwise stated the experiments were carried out at 37°. The final volumes quoted have been corrected to N.T.P. (allowing for the V.P. of water).

The right-hand flask of each apparatus contained 1·5 cc. of xanthine oxidase preparation, 1 cc. of either peroxidase solution or phosphate buffer of p_H 7·6, and the amount of hypoxanthine indicated in the table. The volume was in each case made up to 3 cc. with buffer. The control flasks of the apparatus contained the same amount of enzyme preparation and peroxidase solution as was placed in the experimental flask, the final volume being made up to 3 cc. with buffer. Since the only difference in the contents of the control flask and the experimental flask was, in each case, the presence of hypoxanthine in the latter, any oxygen uptake observed must necessarily be due to the oxidation of hypoxanthine itself or to some secondary oxidation induced by the oxidation of the hypoxanthine.

The apparatus was mechanically shaken in the bath for five minutes before closing the taps in order to establish temperature equilibrium. The initial rates of oxygen uptake in these experiments were found to be almost linear and, hence, in order to allow for the oxidation taking place during this time, the oxygen uptake during the five minutes immediately following the closing of the taps was noted and an equal amount added to the final uptake observed. Four of the instruments could be shaken side by side in the same bath.

All the experiments described were carried out in duplicate, and in nearly every instance the figures quoted are typical of a considerable number of

results. Table I shows the effect of milk peroxidase on the oxygen uptakes of unextracted and extracted xanthine oxidase preparations in presence of hypoxanthine (Hx).

Table I.

No. of Exp.	Contents of flask	Theoretical O_2 uptake for Hx added mm.³	Observed uptake mm.³	Observed uptake with peroxidase mm.³
1	Unextracted preparation + Hx (1 mg.)	165	164	193
	Extracted preparation + Hx (1 mg.)	165	162	168
2	Unextracted preparation + Hx (1 mg.)	165	174	252
	Extracted preparation + Hx (0·91 mg.)	150	151	159
3	Unextracted preparation + Hx (1 mg.)	165	166	215
	Extracted preparation + Hx (1 mg.)	165	176	184

It can be seen from these results that when the extracted enzyme preparation was used, both with and without peroxidase, the oxygen uptake observed was close to the theoretical uptake for the amount of hypoxanthine added. When the unextracted preparation was used without peroxidase, the oxygen uptake observed was again practically theoretical; but when peroxidase was added to the unextracted enzyme preparation plus hypoxanthine, the oxygen uptake was in every instance considerably in excess of the theoretical uptake for the amount of hypoxanthine used. It seems evident from these results that there is present in the unextracted preparation some substance which is oxidised when hypoxanthine is oxidised in the presence of peroxidase, but is not oxidised to any appreciable extent when peroxidase is absent. The fact that the peroxidase is necessary for this oxidation would seem to indicate that the oxidising agent is hydrogen peroxide, the peroxidase serving to activate this. The oxidisable substance appears to be largely removed from the enzyme preparation by thorough extraction with ether, since, when the extracted enzyme preparation is used, the oxygen uptake, either with or without peroxidase, is close to the theoretical for the amount of hypoxanthine used.

In a few exceptional cases we have obtained enzyme preparations which give with hypoxanthine, in the absence of peroxidase, an oxygen uptake somewhat in excess of theoretical, both before and after extraction with ether. The reason for this we have not so far been able to explain.

In experiments, described later in the paper, we found that the oxidation of lactic acid and β-hydroxybutyric acid could be induced if milk peroxidase were replaced by a ferrous salt. Accordingly experiments were carried out to ascertain whether ferrous iron could replace natural peroxidase in the system unextracted enzyme preparation plus hypoxanthine. The results of these experiments are given in Table II, Exps. 1–3, and show that ferrous iron can act like milk peroxidase in bringing about a similar high uptake with the unextracted preparation and that this uptake is reduced after the extraction of the enzyme with ether. Exactly equal quantities of iron as ferrous ammonium sulphate were added, together with xanthine oxidase, to the control flasks of

the apparatus, to eliminate any additional oxygen uptake due to the oxidation of the ferrous salt.

Preliminary experiments showed that in phosphate buffer solution at p_H 7·6, ferrous ammonium sulphate is oxidised very rapidly, and that the greater part is oxidised while the apparatus is attaining temperature equilibrium before the taps are closed. Further, the amount of iron added was equivalent only to a relatively very small volume of oxygen, so that any increased uptake observed in the presence of ferrous iron could not be due merely to the oxidation of the iron.

Table II.

No. of Exp.	Contents of flask	Theoretical O_2 uptake for Hx added mm.³	Iron added	Observed O_2 uptake mm.³
1	Unextracted preparation + Hx (1 mg.)	165	—	160
	" " + " (")	165	+0·14 mg. Fe as ferrous iron	236
2	" " + " (")	165	—	176
	" " + " (")	165	+0·14 " "	214
3	Extracted " + " (")	165	—	155
	" " + " (")	165	+0·14 " "	173
4	Unextracted " + " (")	165	—	175
	" " + " (")	165	+0·03 " as haemoglobin	246
	Extracted " + " (")	165	—	170
	" " + " (")	165	+0·03 " "	174
	" " + " (")	165	+0·06 " "	171
5	Unextracted " + " (")	165	—	163
	" " + " (")	165	+0·03 " "	220
	Extracted " + " (")	165	—	150
	" " + " (")	165	+0·03 " "	175
	" " + " (")	165	+0·06 " "	166
6	Unextracted " + " (")	165	—	157
	" " + " (")	165	+0·22 " as haematin	253
	Extracted " + " (")	165	—	153
	" " + " (")	165	+0·22 " "	216
7	Unextracted " + " (")	165	—	184
	" " + " (")	165	+0·22 " "	249
	Extracted " + " (")	165	—	150
	" " + " (")	165	+0·22 " "	194

Exps. 4–7 in Table II show that both haemoglobin and haematin can act very efficiently as peroxidases in bringing about the secondary oxidation of ether-soluble substances in the unextracted preparation. After the extraction of the preparation with ether, the uptake with haemoglobin in presence of hypoxanthine is reduced practically to theoretical, but with haematin, though the uptake is reduced by extraction, it is still considerably in excess of that given by the extracted preparation plus hypoxanthine alone. It would seem therefore that the oxidation of some ether-insoluble substance or substances occurs in the extracted preparation in presence of haematin. Possibly, under the influence of a strong peroxidase like haematin, an oxidation of uric acid might be taking place. We hope to carry out further investigation on this point. In the case of haemoglobin, the haemoglobin in the flask containing hypoxanthine is very rapidly oxidised to methaemoglobin,

while the haemoglobin in the control flask remains practically unchanged throughout the experiment. This change to methaemoglobin takes place almost completely during the time that the apparatus is attaining equilibrium in the bath before the taps are closed; further, any gaseous exchange involved in the conversion of the small amount of oxyhaemoglobin employed into methaemoglobin would be negligible compared with the uptakes being measured, so that this change cannot account for the increased uptake. That the extra uptake cannot be due to oxidative breakdown of the haemoglobin molecule was shown by taking equal volumes of the solutions in the control and experimental flasks at the end of an experiment, making alkaline with Na_2CO_3 and reducing to haemoglobin with sodium hydrosulphite. After shaking with air, both solutions when viewed spectroscopically gave the bands of oxyhaemoglobin with approximately equal intensity, showing that no appreciable permanent destruction of the haemoglobin molecule had taken place.

Incidentally, this oxidation of haemoglobin to methaemoglobin, which takes place only in the presence of the complete primary system hypoxanthine plus enzyme, affords another example of a secondary oxidation brought about during the oxidation of the primary system. This oxidation, as is well known, is readily brought about by hydrogen peroxide and would therefore be expected in this system.

A similar conversion of oxyhaemoglobin to methaemoglobin by peroxide in sterile extracts of pneumococci is described by Neill and Avery [1924].

The haemoglobin used in our experiments was a 20 % aqueous solution. It was prepared by treating blood corpuscles successively with ether and salt as described by Adair [1925]. The haematin solution was made by dissolving 0·25 g. of pure haematin in three equivalents of 10 % NaOH and making the solution up to 10 cc. with water[1].

During the winter months we have been unable to obtain the additional oxygen uptakes with the unextracted enzyme in presence of milk peroxidase or ferrous iron. It seems possible that this may be due to a change in the food of the cows especially as it is known that the fats found in the milk show a close parallelism with those of the diet.

The additional oxygen uptake obtained in presence of haemoglobin and haematin however has remained unimpaired.

Attempts were made to restore the fat extracted from the enzyme preparation and so produce an increased oxygen uptake with a peroxidase. The greater part of the ether from the extract in the Soxhlet apparatus was boiled off, the concentrated solution of fat then being poured over the extracted preparation and the remaining ether removed *in vacuo*. The resulting preparation gave no increased uptake with ferrous iron or haemoglobin however. Probably the readily oxidisable substances in the fat had already been oxidised by the ether peroxide formed during the extraction.

[1] We wish to thank Mr G. S. Adair for the haemoglobin and Mr R. Hill for the haematin used in these experiments.

Several experiments were carried out using inorganic ferric iron instead of haematin. The iron, in the form of ferric chloride, was added to each flask in an amount equivalent to 0·35 mg. of iron and in no case was any extra uptake of oxygen observed. This inability of free ferric iron to act as a peroxidase might well be expected, since it is incapable of giving the ordinary peroxidase reactions. But it would appear somewhat difficult to explain the peroxidase activity of ferrous iron since the latter, to a large extent at least, is, under the conditions of the experiment, very rapidly oxidised to ferric iron. Possibly during the reaction a small concentration of iron is maintained in the ferrous state by the formation of a complex which can act as a peroxidase in the system.

While it is felt to be unsatisfactory that we have not as yet been able to determine definitely whether these increased uptakes in presence of a peroxidase are actually due to the oxidation of fat, we have nevertheless given the results which we have obtained up to the present, as they appear to have a definite bearing on secondary oxidations.

Oxidation of Lactic Acid.

It has been known for some time that hydrogen peroxide is capable of bringing about the oxidation of lactic acid, especially in the presence of a ferrous salt. Fenton and Jones [1900] showed that, at low temperatures, free lactic acid is oxidised by hydrogen peroxide to pyruvic acid, and Dakin [1908] showed that by distilling ammonium lactate with hydrogen peroxide under reduced pressure acetaldehyde is formed. Since the oxidation of lactic acid plays so important a rôle in physiological processes, it would be of great interest if the oxidation of lactic acid could be correlated with such oxidising systems as the xanthine oxidase plus one of its substrates, or with a non-enzymic oxidation such as that of the sulphydryl compounds.

Experiments were therefore carried out to determine whether the oxidation of lactic acid could be induced during the oxidation of hypoxanthine or xanthine in the presence of xanthine oxidase. The results of some of these experiments are given in Table III.

Table III.

No. of Exp.	Contents of flask	Theoretical O_2 uptake for Hx or xanthine used mm.³	Observed uptake mm.³	Increase due to oxidation of lactic acid mm.³
1	X. oxidase + Hx (1 mg.) + Fe^{++} (0·14 mg.)	165	183	—
	X. oxidase + Hx (1 mg.) + Fe^{++} (0·14 mg.) + 0·4 cc. Na lactate	165	213	30
2	X. oxidase + Hx (1 mg.) + Fe^{++} (0·14 mg.)	165	173	—
	X. oxidase + Hx (1 mg.) + Fe^{++} (0·14 mg.) + 0·4 cc. Na lactate	165	219	46
3	X. oxidase + xanthine (2 mg.) + Fe^{++} (0·14 mg.)	165	165	—
	X. oxidase + xanthine (2 mg.) + Fe^{++} (0·14 mg.) + 0·4 cc. Na lactate	165	206	41
4	X. oxidase + xanthine (2 mg.) + Fe^{++} (0·14 mg.)	165	155	—
	X. oxidase + xanthine (2 mg.) + Fe^{++} (0·14 mg.) + 0·4 cc. Na lactate	165	212	57

The iron was added as ferrous ammonium sulphate, and extracted enzyme preparation was used in these experiments. A number of different samples of lactic acid were used, and all gave similar results. The free syrupy acid was neutralised with 40 % sodium hydroxide, being cooled under the tap during the addition of the alkali. The oxidation of lactic acid was not induced to any appreciable extent by milk peroxidase, but it is evident from Table III that it can be brought about by substituting a small quantity of a ferrous salt for the milk peroxidase. Xanthine appears to bring about the oxidation as readily as hypoxanthine. An equal quantity of ferrous iron was added to the control flask together with lactic acid and, as explained above, the increased uptake observed with lactic acid cannot be due to the oxidation of the iron. It was found that no appreciable oxidation of lactic acid took place unless the latter were present in fairly high concentration. This is understandable, for the hydrogen peroxide formed during the oxidation of hypoxanthine can also serve to bring about the oxidation of further hypoxanthine in the presence of its enzyme, so that we may expect the hydrogen peroxide to be shared between the hypoxanthine and lactic acid (or other secondary substrate), the amount of the oxidation of the latter depending both on its concentration and on the relative ease with which it is oxidised by hydrogen peroxide in the presence of a peroxidase. Thus, though the oxygen uptake due to the oxidation of lactic acid is relatively small, and represents the oxidation of only a small proportion of the lactic acid present, this would nevertheless be expected from the nature of the system as will be further pointed out later on. The relatively high concentration of lactic acid required does not necessarily mean that such an oxidation is unphysiological, for localised high concentrations of lactic acid are certainly present in the living body.

It was shown by Thurlow [1925] that the oxidation of nitrites could also be brought about (in the presence of peroxidase), if the sulphydryl compounds cysteine or glutathione were substituted for the systems xanthine oxidase plus hypoxanthine or xanthine. In their oxidation these two pairs of enzymic and non-enzymic systems appear to be in many ways equivalent. Accordingly experiments were made to find whether the oxidation of lactic acid could be induced during the oxidation of cysteine. The results of these experiments are summarised in Table IV.

Table IV.

No. of Exp.	p_H	Theoretical O_2 uptake for cysteine used mm.³	Observed uptake for cysteine alone mm.³	Lactic acid added cc.	Observed uptake for cysteine + lactic acid mm.³	Increase due to oxidation of lactic acid mm.³
1	6·0	250	260	0·4	313	53
2	6·0	178	144	0·4	232	88
3	6·0	178	145	0·4	272	127
4	6·2	178	163	0·4	267	104
5	6·2	250	254	0·2	319	65
6	6·2	250	252	0·4	349	97
7	7·6	178	186	0·4	236	50
8	7·6	214	209	0·4	270	61
9	7·6	178	179	0·2	239	60
10	7·6	178	180	0·4	274	94

Addition of peroxidase gave no appreciable increase in oxidation of lactic acid, and the oxidation was not much affected by changes in p_H from 6·0 to 7·6.

It is evident from these results that a definite oxidation of lactic acid is brought about during the oxidation of cysteine. Further, this oxidation can take place without the addition of a peroxidase. It has been shown by Warburg and Sakuma [1923] and Harrison [1924] that the oxidation of the sulphydryl compounds cysteine and glutathione is a catalysis by iron, and that preparations of these compounds usually contain iron. It is believed that the iron catalyses the reaction between oxygen and the sulphydryl group by forming a complex with the latter. The presence of a labile ferrous compound is maintained in solution as long as any unoxidised sulphydryl compound remains, and this ferrous compound might well be expected to act as an efficient peroxidase in the oxidation of lactic acid induced by the oxidation of cysteine.

An attempt was made to find what substance or substances are produced by the oxidation of lactic acid.

1 cc. of cysteine solution containing 100 mg., brought to p_H 7·6 with sodium hydroxide, was added to 0·4 cc. of sodium lactate and 8·6 cc. p_H 7·6 phosphate buffer. The solution was shaken in presence of air in a closed flat-bottomed flask at 37° in a water-bath. Two control flasks containing 1 cc. of cysteine solution plus 9 cc. buffer, and 0·4 cc. sodium lactate plus 9·6 cc. buffer respectively, were shaken at 37° for the same length of time. When all the cysteine was oxidised (about 4½ hours) the flasks were cooled and 8 cc. of solution was pipetted from each and distilled into 1 cc. of ice-cold water, the distillation being continued until about 3 cc. had distilled over. Rimini's test for acetaldehyde was then applied by adding two drops of a saturated solution of sodium nitroprusside and two drops of piperidine. The solution which had contained cysteine and sodium lactate gave a positive reaction, whilst the two controls were completely negative. The remainder of each of the solutions was tested for pyruvic acid by the nitroprusside test and the guaiacol test [Quastel, 1924], negative results being in every case obtained. It is thus evident that the product of this secondary oxidation of lactic acid is acetaldehyde. It seems worthy of note that if a similar oxidation of lactic acid occurs when hypoxanthine plus the enzyme preparation from milk replaces cysteine as the primary system, it would be expected that the acetaldehyde formed would be still further oxidised, since the enzyme preparation contains the Schardinger enzyme.

Oxidation of β-hydroxybutyric acid.

Preliminary experiments showed that the oxidation of β-hydroxybutyric acid by hydrogen peroxide in neutral solution at 37° can be detected by applying the nitroprusside test for acetoacetic acid and acetone, and that the oxidation is accelerated by addition of ferrous iron or milk peroxidase.

Experiments were then carried out to find whether the oxidation of β-hydroxybutyric acid could be induced during the oxidation of hypoxanthine plus xanthine oxidase, and during the oxidation of cysteine. The β-hydroxybutyric

acid used in these experiments was a sample from Kahlbaum, and contained no appreciable quantity of acetone or acetoacetic acid. The solution employed was obtained by neutralising the syrupy acid with 40 % sodium hydroxide, cooling under the tap during the addition of the alkali.

The experiments given in the following table were carried out using hypoxanthine plus xanthine oxidase as the primary oxidising system. The solutions were brought to a volume of 3·9 cc. with p_H 7·6 buffer, the iron being added as ferrous ammonium sulphate. After aerating slowly for $3\frac{1}{2}$ hours at 20°, the solutions were saturated with ammonium sulphate, filtered, treated with a few drops of ammonia plus sodium nitroprusside and allowed to stand for about 15 mins. for development of colour.

No. of tube	Contents of tube	Nitroprusside test
1	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 1·5 cc. milk peroxidase	–
2	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 1·5 cc. milk peroxidase + 2 mg. Hx	–
3	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 2 mg. Hx	–
4	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 0·14 mg. Fe^{++}	–
5	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 0·14 mg. Fe^{++} + 2 mg. Hx	++

It is evident from these results that hypoxanthine plus enzyme can bring about the oxidation of β-hydroxybutyric acid in the presence of ferrous iron, but not to any appreciable extent in the presence of milk peroxidase.

Similar experiments were then carried out using cysteine instead of hypoxanthine plus xanthine oxidase. The tubes were aerated slowly until the oxidation of the cysteine was complete and a drop of the solution gave no immediate colour with sodium nitroprusside + ammonia + ammonium sulphate. The solutions were saturated with ammonium sulphate, then filtered, treated with a few drops of ammonia and sodium nitroprusside solution and allowed to stand for about 15 minutes for development of colour.

No. of tube	Contents of tube	Nitroprusside test
1	0·05 cc. β-hydroxybutyric acid + 2·4 cc. milk peroxidase	–
2	2·4 cc. buffer + 20 mg. cysteine	–
3	0·05 cc. β-hydroxybutyric acid + 2·4 cc. buffer + 20 mg. cysteine	+
4	0·05 cc. β-hydroxybutyric acid + 2·4 cc. milk peroxidase + 20 mg. cysteine	++
5	0·025 cc. β-hydroxybutyric acid + 2·4 cc. milk peroxidase + 20 mg. cysteine	+

The experiments were carried out at 30° and buffer was added where necessary to make the volumes of the solution equal to 3 cc.

(The nitroprusside test is given immediately by cysteine even in very dilute solutions, whilst in the case of acetoacetic acid and acetone, at these low concentrations the colour takes about 15 minutes to develop. There is no risk therefore of confusing the reaction for acetone bodies with that given by cysteine.) Controls in which 20 mg. of cysteine in 3 cc. of 7·6 buffer were first allowed to oxidise completely, 0·025 cc. and 0·05 cc. of β-hydroxybutyric acid being subsequently added, gave no appreciable nitroprusside test after shaking at 30° for the same length of time.

After aeration a small quantity of the solution from tube No. 4 gave Arnold's test with *p*-aminoacetophenone for acetoacetic acid.

It is evident that cysteine is capable of bringing about the oxidation of β-hydroxybutyric acid without the addition of peroxidase. A similar result was obtained with lactic acid and, as pointed out above, the presence of ferrous iron in the cysteine solution is probably a necessary factor in these oxidations. It can be seen that the oxidation of β-hydroxybutyric acid by cysteine is increased by the addition of milk peroxidase.

An attempt was then made to estimate the acetoacetic acid produced in this oxidation of β-hydroxybutyric acid. Three closed flasks containing (1) cysteine + β-hydroxybutyric acid + milk peroxidase, (2) cysteine + β-hydroxybutyric acid, (3) β-hydroxybutyric acid + peroxidase, the volumes of each solution being made up to 12 cc. with phosphate buffer of p_H 7·6, were shaken in a bath at 37° until the cysteine was completely oxidised (about 5 hours). The flasks were then cooled and the acetone bodies estimated by the method described by Lublin [1922]. 5 cc. of solution were withdrawn from each flask, diluted with 45 cc. water, 2 cc. of 20 % acetic acid added and the solution distilled for 10 minutes into *N*/100 iodine in alkaline solution. The difficulty in the distillation owing to frothing was overcome by adding 20 g. of ammonium sulphate before distilling. It has been shown by Goldblatt [1925] and others that the presence of proteins does not affect the results in this distillation. The excess of iodine was liberated with sulphuric acid and titrated with *N*/50 sodium thiosulphate (factor = 0·94). The distillations were carried out in duplicate and these agreed well together.

The results are given in the following table:

Contents of flask	Thiosulphate required (for 5 cc. of original solution) cc.		Total aceto-acetic acid formed mg.	Nitro-prusside test
	I	II		
1·0 cc. β-hydroxybutyric acid + 5 cc. peroxidase	13·8	13·7	—	–
1·0 cc. β-hydroxybutyric acid + 5 cc. buffer + 100 mg. cysteine	12·6	12·7	0·35	+
1·0 cc. β-hydroxybutyric acid + 5 cc. peroxidase + 100 mg. cysteine	12·4	12·4	0·43	+ +

Attempts were made to find whether the oxidation of the acetoacetic acid can be induced by cysteine. A quantity of acetoacetic acid sufficient to give a faint but definite nitroprusside test was aerated with 20 mg. of cysteine in 2·5 cc. of milk peroxidase. No appreciable diminution in the intensity of the nitroprusside test could be observed however.

Discussion.

From the work of Thurlow [1925] it was established that a peroxide is formed during the oxidation of hypoxanthine and xanthine (in presence of the xanthine oxidase), and since such systems as oxidising cysteine and oxidising

glutathione were shown to behave in the same way in bringing about the oxidation of nitrite when peroxidase was added, it was pointed out that a peroxide is probably formed in these systems also. Strong evidence was given for the belief that in the cases of hypoxanthine and xanthine the peroxide formed was hydrogen peroxide rather than an organic peroxide. In the case of oxidising cysteine and oxidising glutathione no definite assertion was made as to the nature of the peroxide formed, though it was pointed out that by assuming the formation of hydrogen peroxide rather than an organic peroxide, the mechanism of the oxidation of sulphydryl compounds might be explained in a manner more nearly comparable to that of the other oxidising systems. It may be mentioned that Engler and Broniatowski [1904] obtained, under favourable conditions, a yellow colour with titanium sulphate during the oxidation of thiophenol and therefore believe that hydrogen peroxide is formed during the oxidation of the sulphydryl group.

Recently it has been shown by Dixon [1925] that the destruction of xanthine oxidase which occurs during the oxidation of hypoxanthine can be inhibited by the addition of catalase; and, since the enzyme is destroyed by hydrogen peroxide and since catalase acts apparently only on hydrogen peroxide and not on organic peroxides, this is further evidence for the formation of hydrogen peroxide in the hypoxanthine system.

In the present paper it has been shown that the secondary oxidation of lactic acid and β-hydroxybutyric acid brought about during the oxidation of hypoxanthine occurs only in presence of a peroxidase. This again supports the view that hydrogen peroxide is formed, since organic peroxides are capable of bringing about secondary oxidations without the addition of a peroxidase. For example, it is well known that organic peroxides are capable of blueing guaiacum and benzidine directly and Dixon (unpublished experiments) has found that benzoylhydrogen peroxide can oxidise nitrite without peroxidase.

It would have been of interest to ascertain whether the secondary oxidations brought about by cysteine require the presence of a peroxidase. Unfortunately this is difficult to prove, for it has been shown by Warburg and Sakuma [1923] and by Harrison [1924] that the oxidation of sulphydryl compounds is dependent on the presence of traces of iron which these compounds normally contain. This iron might well be expected to act as an efficient peroxidase. If the iron be removed however by very careful purification, or inactivated by cyanide, the oxidation of the sulphydryl group is very greatly inhibited and consequently it would be very difficult to detect any secondary oxidation should it occur.

The work of Dakin has given strong support to the view of the formation of peroxides during biological oxidations by showing the very close similarity which exists between the oxidations which can be brought about *in vitro* by hydrogen peroxide and those actually occurring in the body as determined by metabolism experiments. On the other hand, there appears to be no such parallelism in the oxidations brought about by the dehydrase type of mech-

anism; for example, the oxidation of succinic acid to fumaric acid, and of hypoxanthine to uric acid are typical dehydrase oxidations and are not brought about by hydrogen peroxide, whilst the oxidation of, say, the higher fatty acids is readily brought about by hydrogen peroxide and not by dehydrases. This appears to support the view that oxidations which Dakin has shown can be imitated by hydrogen peroxide *in vitro* do not take place directly by a dehydrase mechanism, but by secondary oxidation by means of peroxide on the lines described in the present paper.

The wide distribution of catalase in the living organism cannot necessarily be regarded as evidence against the occurrence of oxidations brought about by hydrogen peroxide in presence of peroxidase, for it has been shown by Chodat and Pasmanik [1907] that a partition of hydrogen peroxide between catalase and peroxidase occurs when both enzymes are present. Further, Thurlow [1925] has shown that catalase only partially inhibits the secondary oxidation of nitrites.

From the results given in Tables I–IV it can be seen that the increased oxygen uptake due to secondary oxidation is never as great as the uptake of the primary system alone, though in some cases it approaches this value. The total uptake in presence of both the primary and the secondary system is never greater than double that of the primary system alone. This is a result which would be expected if the secondary oxidation is brought about by a peroxide on the lines indicated earlier in the paper. A molecule of oxygen taken up during the oxidation of two molecules of, say, cysteine, would form one molecule of peroxide, and one atom of oxygen in this peroxide would then be available for bringing about secondary oxidation. Thus under the most favourable conditions the oxygen absorbed would be divided equally between the primary and secondary systems. On the other hand, if the secondary system were absent, the one molecule of peroxide would bring about the oxidation of a further two molecules of cysteine, the primary oxidisable substance. In any case, even in presence of the secondary system we should expect some of the peroxide oxygen to be used by the primary system and consequently the oxygen uptake due to the secondary oxidation would always be less than that due to the primary oxidation, a result which agrees with our experiments.

Hopkins [1925] has obtained a similar result in studying the induced oxidation of unsaturated fatty acids by glutathione in neutral solution[1]. Here again the oxygen uptake ceases when the total uptake is nearly double that of the sulphydryl compound alone. It would seem probable that this induced oxidation of the fatty acids is being brought about on the lines suggested above, in a similar way to the induced oxidation of lactic acid.

The induced oxidation of proteins by sulphydryl compounds recently described by Hopkins [1925] presents a difficulty, for in this case the oxygen

[1] At a decidedly acid p_H of about 3·5 the oxidation of the fatty acids appears to be induced by an entirely different mechanism, glutathione here acting catalytically as an oxygen carrier apparently in much the same way as iron acts.

uptake in presence of protein may amount to as much as ten times the oxygen uptake of the sulphydryl compound alone. A parallel case would seem to be that of the induced oxidation of linseed oil by glutathione, where the total oxygen uptake is many times greater than that of the sulphydryl compound alone. It is possible of course that the induced oxidation of proteins and linseed oil takes place by means of a different mechanism from that of the reactions which we have been considering. An alternative possibility, however, would seem to be that, in the case of protein and linseed oil, secondary oxidation is brought about by peroxide during the oxidation of the sulphydryl group in the manner described above, and that the oxidation products of the protein and linseed oil then spontaneously take up more oxygen in a reaction or series of reactions involving their more complete oxidation. The fact that, as shown by Dakin, the individual amino-acids are readily oxidised by hydrogen peroxide would make it not improbable that the secondary oxidation of proteins might be brought about on such lines.

The work of Hopkins has established a link between the oxidation of fats and the oxidation of proteins. The proteins containing a sulphydryl group reduce oxidised glutathione and the reduced glutathione then brings about the oxidation of fats. The reducing factor in the protein regulates the supply of the soluble sulphydryl compound in the tissues and is thus the controlling factor in this oxidation of fats. In the present paper we have shown that the oxidation of lactic acid and β-hydroxybutyric acid is brought about at a physiological p_H during the oxidation of the sulphydryl group, and consequently these oxidations will also be controlled by the sulphydryl groups of the proteins in the tissues. Since lactic acid is one of the most important substances concerned in the intermediary metabolism of carbohydrates, this establishes a link between the oxidation of proteins and the oxidation of carbohydrates in the living cell.

The mechanism of secondary oxidation described in this paper would form an alternative means for the oxidation in the living cell of substances such as lactic acid which are also oxidised by loss of hydrogen in presence of their specific enzymes or "hydrogen transportases" to use Thunberg's terminology. On the other hand, it might be a means of oxidation of substances such as the sugars for which no hydrogen transportase has been found in the tissues. Such a peroxide mechanism would probably be much less specific than the dehydrase oxidations, the peroxide oxygen being shared among the various oxidising substances in proportion to their concentrations, the relative ease with which they are oxidised and the speed with which their oxidation products are removed.

At the present time we have only studied the secondary oxidation of a few substances, and there seems little reason to doubt that the oxidation of many other substances may be brought about in a similar way. In using the Barcroft technique it is not possible to detect secondary oxidations unless they occur to a considerable extent, for small differences in oxygen uptake have to be

neglected since the experimental error is considerable. In the body the conditions may very possibly be more favourable, and other factors such as surface may well play a part. Further, it seems likely that a number of different substances undergoing oxidation together may mutually assist one another. For example, it has been shown by Shaffer [1921] that the oxidation of acetoacetic acid by hydrogen peroxide is much accelerated in the presence of glucose.

It is very unlikely that these secondary oxidations would be prevented owing to lack of a suitable peroxidase, for, apart from the widely distributed animal peroxidases, it has been shown that haemoglobin can act as a very efficient peroxidase in these reactions.

In this connection the respiratory pigment cytochrome, recently described by Keilin [1925] and shown to give the ordinary peroxidase reactions, may well be an important factor.

In the tissues there are present very many "primary" oxidising systems besides those we have studied. Thurlow [1925] has shown that on adding peroxidase, the secondary oxidation of nitrites can be induced during the oxidation of succinic acid in presence of succinoxidase. Doubtless many other oxidising systems are capable of inducing secondary oxidations to a greater or less extent in a similar manner, and the combined action of these various primary systems might well be expected to bring about a very considerable amount of secondary oxidation in the living organism.

Summary.

1. During the aerobic oxidation of hypoxanthine in presence of xanthine oxidase, the oxidation of some substance present in milk, possibly a fat, can be induced if a suitable peroxidase is added.

2. Milk peroxidase, ferrous iron, haemoglobin and haematin can act as peroxidases in this oxidation.

3. The oxidation of lactic acid to acetaldehyde and of β-hydroxybutyric acid to acetoacetic acid can be induced during the aerobic oxidation of cysteine.

4. The aerobic oxidation of hypoxanthine in presence of xanthine oxidase can induce the oxidation of lactic acid and β-hydroxybutyric acid if ferrous iron be added.

5. The bearing of these results on the mechanism of biological oxidations is discussed.

We wish to express our thanks to Sir F. G. Hopkins for his continued interest and encouragement in this work.

One of us (D. C. H.) wishes to express his indebtedness for a Keddey Fletcher-Warr Studentship, University of London, held during the course of this work.

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XXIX. THE EFFECT OF COPPER COMPOUNDS UPON THE GROWTH OF CARCINOMA IN THE RAT.

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Sugiura and Benedict [1922] tested the effect upon the Flexner-Jobling rat carcinoma of 32 salts of a large number of metals (Na, K, Cu, As, Mg, Zn, Hg, Ca, Sr, Ba, Mn, Fe, Co, Mo, W, U, Se, Te, Ge, Pb, Zr, Os) given by the mouth. Of these 22 metals, copper showed the most definite inhibitory action upon the growth of the tumour. The metal was given as a watery solution of "$CuSO_4$" of strength up to 0·5 % mixed with the food; "each day a mixture of 50 parts of the basal diet and an equivalent of the salt solution was prepared."

The experiments described below consisted in the administration of copper by the method of Sugiura and Benedict to rats bearing an inoculated adenocarcinoma (Rat tumour 9 of the Imperial Cancer Research Fund), obtained through the kindness of Dr J. A. Murray. The original tumour of this strain arose in the abdomen, but the exact site of origin could not be determined.

Experimental.

The copper compounds were administered by the mouth. 100 cc. of solution of copper salt were mixed with 100 g. biscuit meal, which is then of a distinct greenish-blue colour. The copper solutions used were as follows: (*a*) $CuSO_4.5H_2O$ in 0·3 % solution. The rats did not appear to be in good health when given a stronger solution (0·4 %). (*b*) Copper glycine, 0·25 %. (*c*) The solution obtained by mixing solutions of NaOH and $CuSO_4$ in the presence of sufficient cane sugar to prevent precipitation of cupric hydroxide ((*b*) and (*c*) were equivalent in copper content to (*a*)). The control rats received the same food but without added Cu. The duration of the experiment was usually from 2 to 3 months, depending upon the rate of growth of the tumour. In some series copper was given for 1 or 2 weeks before the grafting of the tumour.

When the rats were killed the alimentary canal from pharynx to anus was dissected out and rejected to avoid any contamination by copper in the food, the controls being treated in the same way. The bodies were minced, the tumours simply cut up with scissors, the mince being placed in silica dishes, dried at 110° and ashed over a Meker burner with flame collar. Unburnt carbon was oxidised by evaporating the ash to dryness with 5 cc. HCl and

5 cc. HNO_3; the residues were then brought into solution (cloudy) with just sufficient HNO_3 (bodies) or dil. H_2SO_4 (tumours). The solution, made up to 30–60 cc. and heated to 50–70°, was electrolysed at 2–2·5 volts in a 100 cc. beaker, the solution being stirred by a motor-driven glass paddle. Each electrode was of stout Pt foil, flag-shaped, 1 cm^2., joined to a Pt wire. A serviceable electrode surface was obtained by treating the sand-blasted electrode successively with the finest brown emery powder, then with fused $KHSO_4$ and either boiling H_2CrO_4 or preferably HNO_3 and distilled water, and finally flaming at red heat. The copper deposited was removed from the cathode by raising it quickly from the solution with the current unbroken, rinsing with distilled water, and plunging into 5 cc. of a hot mixture of equal volumes of concentrated HNO_3 and water; this must be done as rapidly as possible or re-solution of the copper will occur. Electrolysis was considered to be at an end when no visible change on the surface of the cleaned electrode was observed after 1 hour's further passage of the current. The solution in HNO_3 was evaporated, ignited, and the residue dissolved in 1 cc. dil. H_2SO_4, made up to 10 cc., and spun (Solution A). The residual fluid in the beaker was poured into excess of hot ammonia solution, the precipitated calcium phosphate filtered off, the solution evaporated to dryness, the evaporation repeated with 1 cc. H_2SO_4 to decompose nitrates, the residue dissolved in 1 cc. dil. H_2SO_4, made up to 10 cc., spun, and the clear liquid (Solution B) used for colorimetric estimation of the trace of Cu not removed by electrolysis; this amount was seldom greater than 0·02 mg.

The estimation was made colorimetrically by the potassium ethyl xanthate method. Amounts of standard $CuSO_4$ solution containing from 0·0025 to 0·0125 mg. Cu were placed in tubes (20 × 50 mm.) and made up to 5 cc. with addition of 7 drops of 0·2 % fresh potassium ethyl xanthate solution. Larger amounts of Cu cause turbidity, whilst even much weaker solutions, in the presence of electrolytes and especially of oxidising agents such as nitrates, quickly become cloudy. For this reason separate colour comparisons were made with Solutions A and B since the latter must contain the salts of the ash not precipitated by ammonia. Experiments showed that the maximum change in colour intensity was obtained within the range of concentration 0·005 to 0·009 mg. Cu in 5 cc.; accordingly amounts were taken from the solutions A and B which would give a concentration of Cu within this range. The best form of comparator was found to be a box backed with thin white paper; ground-glass is unsuitable. The match must be made immediately after adding the xanthate before turbidity appears. Results for one ash (Solution A + Solution B) obtained by two independent observers seldom differed by more than 10 %.

The accuracy of this method was tested as follows. The ash from the body of a rat which had received injections of Cu glycine was dissolved with HNO_3 and made up to 100 cc.; to 50 cc. of this was added $CuSO_4$ solution equivalent to 0·075 mg. Cu (*i.e.* about $\frac{1}{4}$ of the copper content of the rat). The Cu content

of the two portions was then estimated in the manner adopted throughout these experiments: the Cu was recovered quantitatively.

	Mg. Cu found
50 cc. ash solution + added Cu	0·230
50 cc. ash solution alone	0·155
Difference	0·075

Seven blank estimations upon the reagents used in an estimation gave the following results: 0·018, 0·017, 0·027, 0·016, 0·020, 0·008, 0·020 mg. Cu, mean 0·018. This amount was subtracted from the results throughout. All the figures given in Table I are calculated from the mean of readings made by two observers independently.

Table I.

		Mg. Cu in 100 g. dry tissue	
		Bodies[1]	Tumours[2]
I. Control Rats			
	Rat 1	0·41	1·9
	2	0·29	1·6
	3	0·67	1·3
	4	0·42	2·7
	5	0·93	2·7
	6	0·40	2·9
	7	1·6	1·8
	8	2·2	—
	9	3·1	—
Mean		1·11	2·13
Mean of Nos. 1, 2, 3, 4, 5, 6		0·52	—
II. Copper-fed Rats			
(*a*) $CuSO_4$-fed	10	0·59	3·2
	11	0·58	1·8
	12	1·3	4·7
	13	1·8	4·4
	14	1·0	—
	15	0·32	—
	16	0·96	—
(*b*) Cu glycine-fed	17	0·61	3·2
	—	—	3·6
	—	—	4·5
(*c*) Cu cane-sugar-fed	—	—	2·9
Mean		0·90	3·54

[1] *I.e.* body after removal of tumour and alimentary canal.

[2] Each figure in this column refers to an analysis of the pooled tumours of several rats.

Discussion.

The figures in Table I show that the average amount of Cu in the body apart from the gut and tumour cannot be greatly increased by giving Cu with the food; the copper-fed rats contained a mean of 0·9 mg. Cu %, and the whole series of controls actually more, namely, 1·11 mg. %. However, the control series includes three rats (Nos. 8, 9, 10) which have a very much higher Cu content than any of the others; no reason for this could be found. If these three be excluded, the mean of the control series is 0·52 mg. Cu %. The figures indicate, then, that the amount of Cu in the body at any one moment is not raised

very much even by food which is coloured greenish-blue with Cu salt; the body must therefore be very well protected against absorption of the metal. Even if the lower figure (0·52 mg.) be taken as correct for the normal, the increase in the copper-fed (to an average of 0·9 mg.) is an increase of only 75 %.

The tumours show a distinct difference between the two series; the tumours of the controls contain on an average 2·13 mg. Cu %, and those of the copper-fed rats 70 % more than this, or 3·54 mg. The figures in each series of tumours show much less wide variations than do those for the bodies of the rats; only one tumour in the copper-fed series contains less copper (1·8 mg. %) than the maximum amount in the controls.

From the therapeutic point of view the important question is whether copper absorbed into the body reaches a higher concentration in the tumour than in the other tissues. The averages in Table I as they stand indicate that the dried tumour of the copper-fed rats contains four times as much copper as the other dried tissues (3·54 : 0·9), and if the lower figure (0·52 mg.) be taken for the normal rats, their tumours and other tissues show just the same ratio (2·13 : 0·52, or 4 : 1). The amounts given in Table I are reckoned on the dried tissue, and the question must be considered whether such results are best expressed as percentages of the dry or of the fresh weight. The fresh body of the rat minus the tumour and gut contains a much higher percentage of solids (about 32 %) than does the tumour (average about 12 %, see Table II): the presence of adipose tissue and bone in the bodies may account for a large part both of this difference in water content, and of the difference in copper content shown by the bodies and tumours (Table I). The figures given below (Table II) show that the percentages of water in a series of samples of tumour tissue show no constant relationship to the amounts of Cu.

Table II.

	Copper-fed					Controls		
Tumour tissue	1	2	3	4	5	6	7	Mean
Total solids in 100 g. of fresh tissue, g.	12·0	22·0	8·7	12·0	7·7	6·0	13·0	11·8
Cu in 100 g. dry tissue, mg.	3·2	3·2	2·9	2·7	4·5	2·9	2·7	—

One may conclude, then, that the results are best expressed as percentages of the dried tissues, as in Table I.

In some of the earlier series of experiments with copper sulphate and copper glycine the metal seemed to have a definite inhibitory effect upon the tumour; an example is given in Table III. The rats were killed as soon as the skin over the tumour appeared likely to break; as there was no other cause of death in this experiment the time of survival after inoculation gives a rough numerical measure of the growth of the tumour.

The table shows a very distinct difference between the two series, especially about the 38th day, when 10 of the controls and only 3 of the copper-fed rats had been killed. Unfortunately in several later experiments the results have been less satisfactory. Large numbers of rats are necessary in this work in

order to correct for natural variations in susceptibility to the tumour. The data given in Table III probably show the maximum inhibition of this particular tumour which can be brought about by increasing the copper in it in the ratio of 2·1 to 3·5 (Table I) or by 66 %.

Table III.

Days after inoculation with carcinoma	Number of rats alive	
	Copper glycine-fed	Controls
0	15	15
34	15	9
38	12	5
52	7	3
88	7	3

The amounts of copper which can be absorbed from the alimentary canal seem then to be insufficient to exert any constant and considerable inhibiting effect upon this carcinoma. The exposure to X-rays of tumours containing such an increased amount of Cu as can be obtained by feeding (3·5 mg. % of dried tissue) would be of interest, as the metal would no doubt give rise to secondary radiations.

SUMMARY.

1. An electrolytic method for the estimation of amounts of copper in tissues of the order of 0·03 to 1 mg. is described.

2. A rat bearing a carcinoma and receiving no addition of copper to the food shows from two to four times as much copper % in the tumour as in the rest of the body, reckoned on the dry weight. The difference is perhaps due in part to the less cellular tissues (fat, bone) in the body.

3. When copper compounds (copper sulphate, copper glycine and copper sugar compounds) are given to carcinoma-bearing rats with the food for periods of 2–3 months, (1) the average amount of copper in the body is not increased by more than 75 %; large individual variations make it difficult to obtain exact results on this point; a few normal rats contain more copper than the average amount found in copper-fed rats: (2) the average amount of copper in the tumour can be increased by about 66 %. The ratio between the amounts of copper in the tumour, and in the rest of the body, is therefore not altered distinctly by giving copper with the food.

4. In some series of rats the administration of copper with the food appears to check the growth of carcinoma, but this effect is not obtained constantly.

This work was done during the tenure of a grant from the British Empire Cancer Campaign. I wish to thank Dr Leitch, the Director of this Institute, for allowing me the use of its facilities. My thanks are due to Dr E. L. Kennaway, who carried out all the experiments on living animals and to whom I am also indebted in many other ways.

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XXX. THE INHIBITORY EFFECT OF THE SUGARS ON SAPONIN HAEMOLYSIS.

By ERIC PONDER and WALTER PHILLIPS KENNEDY.

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(*Received February 3rd, 1926.*)

One of us [Kennedy, 1925] has already made a short study on quantitative lines of the effects of glucose, fructose, and galactose on saponin haemolysis. The general effect was found to be an inhibitory one. In view of the more complete analysis of inhibition of haemolysis made in a recent paper [Ponder, 1926], we have thought it a matter of interest to extend the investigations into the inhibitory effect of the sugars, both with a view to enlarging their scope, and of bringing them into line with the results of the last-mentioned paper.

Material used.

With the exception of the specimens of glucose, fructose, and galactose, the sugars used in this investigation were those of the "Difco" brand. Each sugar was dissolved in distilled water to make a few cc. of a $M/3{\cdot}6$ solution, which is isotonic with the cells employed. In each case the p_H of the solution was determined, and the sugar used only if no acidity was found; in several cases we had to reject samples because they were acid.

The red cells used were those of man and of the sheep. A suspension was prepared by suspending the thrice-washed cells from 1 cc. of blood in 20 cc. of 0·85 % NaCl. In the case of the sheep cells, the blood was received into oxalate instead of into citrate as in the case of the human cells, since citrate solutions which are isotonic for the cells of man are not isotonic for the cells of the sheep.

The saponin (quillia saponin, Merck) was dissolved in sufficient quantities of 0·85 % NaCl to give a series of dilutions from 1 in 1000 to 1 in 70,000.

Technique.

The methods used were those described in several previous papers, and especially that of Ponder [1926]. In some cases complete curves were worked out and analysed, but in the majority of cases it has been thought enough to ascertain whether the particular sugar, in 5 % solution, is inactive, accelera-tory, or inhibitory. This restriction has been necessary owing to the expense attached to a more complete investigation. All experiments were carried out at 25°.

Results.

Glucose. The investigation of the effect of this sugar shows the general procedure adopted. The time-dilution curve for saponin acting on the suspension of human cells, and that of saponin acting on the sheep cells, are first plotted. Saponin solutions, each 2·5 times as concentrated as the concentration desired, are taken, and of each 0·8 cc. is mixed with 0·8 cc. of saline; to the mixture is then added 0·4 cc. of the suspension, precautions being taken as regards the temperature. The curves are shown in Fig. 1. where a different scale is used for the two suspensions; in the case of the sheep cells, the curve lies between saponin dilutions of 1 in 1000 and 1 in 80,000, while in the case of the human cells the curve lies between 1 in 10,000 and 1 in 60,000.

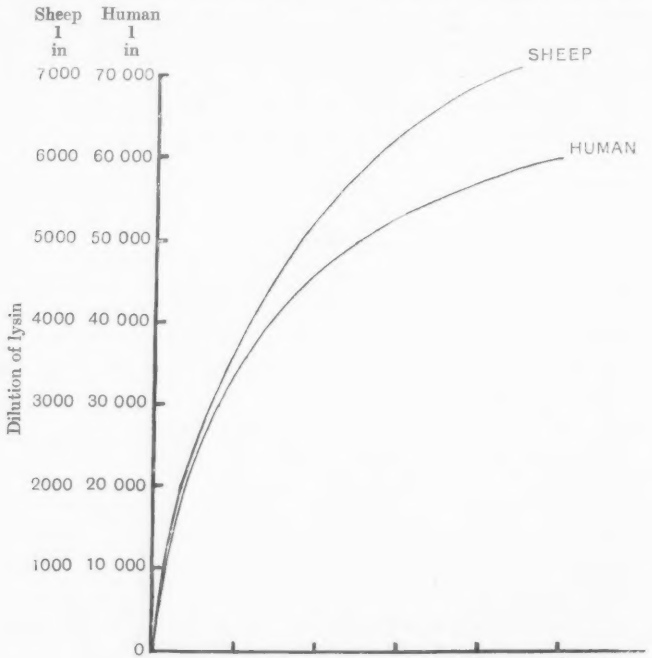

Fig. 1.

The first point which arises is that the two curves are very similar in form, so that we should expect some simple relation between them. This relation is readily made out, and in the following way. We take a point on the curve for sheep cells, say the point (3000; 1·3), or, if the dilution be converted into the number of milligrams of saponin in the 2 cc. contents of the tube, the point (0·66; 1·3). Dropping an ordinate through this point, we get, on the curve for the human cells (27,000; 1·3) or (0·074; 1·3). Dividing the

two concentrations needed to haemolyse in the same time, that is, 0·66 by 0·074, we get the ratio, 8·9. And so with any number of other points. The ratios are:

Sheep cell curve, concentration	0·66	0·5	0·4	0·33	0·286
Ratio	8·9	8·8	8·8	8·6	8·5

Put into words, this means that the sheep cells require 8·8 times the amount of saponin required by the human cells to give lysis in the same time, or, that the sheep cells are 8·8 times as resistant to the action of the saponin as are the human cells. This is a method of analysis which may be used quite freely, and is, in our opinion, the best method of expressing relative resistances.

The time-dilution curve for saponin plus 5 % glucose is now determined by adding to each of the concentrations of saponin, not 0·8 cc. of saline, but 0·8 cc. of 5 % glucose. To the mixture 0·4 cc. of cells is added. Taking the human curve in full, and calling the dilution of saponin used δ_1, its corresponding concentration c_1, the dilution on the curve for saponin alone which has the same time for lysis as is observed in the glucose saponin mixture δ_2, its corresponding concentration c_2, and the difference between c_1 and c_2, x, we get the following table:

δ_1	c_1	δ_2	c_2	x
25,000	0·080	31,000	0·065	0·015
30,000	0·066	36,000	0·055	0·011
40,000	0·050	46,000	0·043	0·007
50,000	0·040	56,000	0·035	0·005

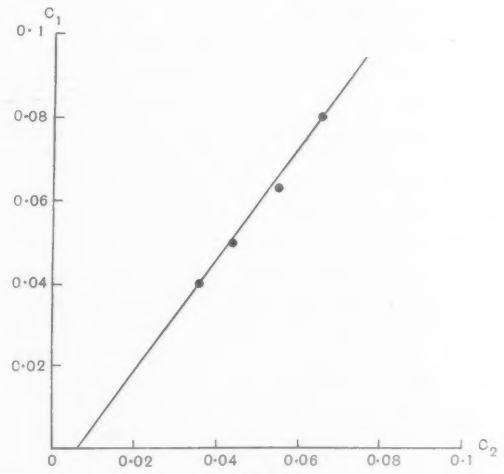

Fig. 2.

Plotting x against c_2 we get a very good straight line; we therefore follow the procedure adopted in similar cases [Ponder, 1926] and plot c_1 against c_2. This also gives a straight line (Fig. 2), which makes an intercept on the c_2-axis of 0·006, and which is described by the expression $c_1 = 1{\cdot}4\ (c_2 - 0{\cdot}006)$. This linear

relation between c_1 and c_2 is similar to that described in the paper last mentioned in connection with the inhibitory effect of such substances as the alkalis; it corresponds to the general expression for these inhibitions, $c_1 = R(c_2 - k)$. It has already been shown that R measures the resistance of the cells in the cell-saponin-glucose system as compared with that of the cells in the cell-saponin system; under the action of the 5 % glucose this resistance is increased 1·4 times. The quantity of glucose actually present in the system, it may be noted, is 40 mg. We have commented on the significance of the constant k elsewhere; for our present purpose it may be disregarded.

The results previously given by Kennedy in a consideration of the inhibitory action of glucose can be treated in the same way as the above. They give $R = 1{\cdot}56$, and $k = 0{\cdot}003$. The action of the glucose was thus to increase the resistance of the cells in the glucose-saponin-cell system 1·56 times. When published, the results were not treated as we treat them now, and the equation applied was one applicable to quite another type of inhibition. The figures, however, are substantially correct, and it will be noted that Kennedy obtained in the expression which he used a value of n which is nearly equal to unity, as required by the above linear relation.

Sucrose. We have found that a $M/3{\cdot}6$ solution of sucrose has an inhibitory effect on saponin haemolysis; if the results are treated as above they yield $R = 1{\cdot}55$, $k = 0{\cdot}005$. These values are not greatly different from those for glucose, and we may therefore conclude that the effect of the two sugars in equimolar solution is very similar.

Lactose. A similar treatment of the inhibitory effect of $M/3{\cdot}6$ lactose gives the following values: $R = 1{\cdot}4$, $k = 0{\cdot}004$. These figures are substantially the same as those for the same concentration of glucose.

The rarer sugars. In the cases of the rarer and less easily obtained sugars, we have contented ourselves with the determination of a single point instead of the entire time-dilution curve. So as to work at the same place on the curve for human cells as for sheep cells, we use a dilution of 1 in 5000 saponin for the latter and a dilution of 1 in 44,000 saponin for the former, the ratio of these dilutions being the same as the ratio of the cell resistances. To 0·8 cc. of the saponin is added 0·8 cc. of the solution of sugar to be examined, and thereafter 0·4 cc. of the proper cell suspension. The quantity of lysin, x, apparently inactivated by the sugar, is found as in the previous instances. A table of the results shows the relative effect of the sugars.

Sugar	Human cells x, mg.	Sheep cells x, mg.
Glucose	0·006	0·07
Mannose	0·008	0·07
Lactose	0·010	0·08
Sucrose	0·013	0·20
Trehalose	0·016	0·04
Maltose	0·013	0·07
Raffinose	0·010	0·10
Arabinose	0·002	inert
Xylose	0·002	inert
Rhamnose	0·004	0·05

From this table several points arise. (1) All the sugars, except arabinose and xylose with sheep cells, are inhibitory. We have been unable to find any instance of acceleration with a sugar, unless the sample was acid in reaction. Kennedy has described acceleration with galactose, but, as the specimen was acid, the observation is to be withdrawn. Samples of inositol, melezitose, and sorbitol which we examined produced acceleration, but these samples were acid, and therefore useless for our purpose. (2) With the human cells, as with the sheep cells, there are considerable differences in the inhibitory effects of the sugars. It is to be remembered that in this table only single points on the curves for the action of the saponin-sugar mixtures are determined, and that we have no values for the respective constants, k. Only if we take it that k is the same in all cases is it correct to take the figures of the table as indicating the respective resistances of the various cell-saponin-sugar systems; k, however, seems to vary little, and so the assumption that it is constant will result in little error. (3) The effects of the sugars on the sheep and on the human cells do not run parallel. If the effect on the former were the same as that on the latter, we should expect the value of x in the latter case to be 8·8 times as great as in the former, for the sheep cells are 8·8 times as resistant as are the human cells. This condition is approximately fulfilled in the cases of glucose, mannose, lactose, arabinose and xylose, and we may therefore take it, provisionally, that there is no great difference between the action of these sugars on the sheep cells and on the human cells. With the other sugars no such assumption can be made.

We do not wish at this stage to offer any explanation as to the mechanism of the inhibition. It is possible that the sugars affect the cells themselves, and we have already dealt with this possibility [Ponder, 1926]. Other possibilities also present themselves; the lysin may be affected, or the reaction between the cells and the saponin may be modified. The method which we have used takes no account of these things; it merely compares the haemolytic systems, irrespective of which component is altered. Nor is it to be lost sight of that when sugar is added to the cell-saponin system, a certain amount of NaCl is of necessity replaced, and the cell-saponin-sugar system is poorer in this salt. This arrangement is necessary in order to maintain the correct tonicity in the system, but the withdrawal of NaCl may in itself create a new haemolytic system which differs from the one taken as a standard. We intend to investigate these points at a later date.

Summary.

(1) The inhibitory action of glucose on saponin haemolysis is studied quantitatively, and the inhibition shown to conform to a type already described in connection with sucrose, certain alkalis, and certain amino-acids.

(2) A number of other sugars were found to be inhibitory in different degrees when human cells are employed in the haemolytic system; when sheep

cells are employed, xylose and arabinose were found to be inert. The action of the various sugars on the human and the sheep cells was found to differ in some cases. No sugar which produced acceleration was met with, unless acidity was present in the sample.

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XXXI. THE INFLUENCE OF NEUTRAL SALTS ON HAEMOLYSIS.

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(*Received February 19th, 1926.*)

In a recent paper it was pointed out that in experiments on the kinetics of haemolysis it is of prime importance to have the tonicity of the system adjusted with strict accuracy. It has been thought that experimental evidence on this point would be desirable, and that at the same time the effects of various kations on saponin haemolysis should be investigated, since variations in the suspension medium also affect the rate of haemolysis and therefore frequently invalidate the comparison of different sets of results.

Method.

The method falls into two parts, the purely manipulative and the mathematical analysis. Detailed descriptions of these will be found in papers by Ponder [1926], and by Ponder and Kennedy [1926]. It is only necessary to add that the chemicals used were B.D.H. analytical reagents and that in the experiments with various ions the system consisted of 0·4 cc. blood suspension in 0·85 NaCl + 0·8 cc. of a $M/6{\cdot}88$ solution of the salt to be examined + 0·8 cc. saponin in varying dilution in 0·85 % NaCl. The principal point of the analysis is that if a first time-dilution curve for a saponin-cell-NaCl system be drawn together with a second curve for a system in which another salt replaces the NaCl, then a straight line may be obtained by plotting the ordinate c_1 of the second curve against the ordinate c_2 of the first curve; and this line is represented by the equation $c_1 = R\,(c_2 - k)$, where R is the slope of the line and k an empirical constant, usually small. In the case of accelerations R is less than 1 and k is negative; in the case of inhibitions R is greater than 1 and k is positive. R affords a measure of the amount of acceleration or inhibition, and the values of R for the various ions can be compared legitimately as they stand, although for strictly quantitative considerations Ponder [1926] should be consulted.

It may be mentioned that there is no difficulty in obtaining these lines from the experimental results, which show negligible deviations as can be seen from the figures.

Results.

Table I. *Effect of varying tonicity on saponin haemolysis.*

	Tonicity of system				
	0·85 %	0·76 %	0·69 %	0·595 %	0·51 %
R	1·0	1·2	1·27	1·29	1·3
k	0·0	0·006	0·006	0·006	0·006

Table II. *Relative effects of kations on saponin haemolysis.*

Salt added to system	p_H of salt solution	Sheep		Cat		Human		Rabbit	
		R	k	R	k	R	k	R	k
LiCl	6·3	0·9	−0·01	1·4	0·04	1·2	0·003	1·0	0·0
$MgCl_2$	6·5	0·77	−0·035	0·6	−0·02	0·74	−0·0045	0·9	−0·001
NaCl	6·2	1·0	0·0	1·0	0·0	1·0	0·0	1·0	0·0
KCl	5·58	0·85	−0·02	0·7	−0·05	0·9	−0·0015	1·0	0·0
$CaCl_2$	6·34	0·77	−0·035	0·35	−0·06	0·74	−0·0035	1·4	0·004
RbCl	5·28	0·85	−0·02	0·69	−0·04	0·82	−0·001	1·0	0·0
$SrCl_2$	6·24	0·85	−0·02	0·50	−0·02	0·74	−0·0035	0·9	−0·001
$BaCl_2$	6·24	0·93	−0·05	0·60	−0·02	0·9	−0·0015	1·0	0·0

It will be noted from the above that k in the case of the sheep and cat is in general some ten times greater than in the human and rabbit. This may be connected with the greater resistance of the former to the haemolytic action of saponin. To minimise experimental error it is expedient to choose a series of dilutions of saponin such as will give end points in a time range of half to 6 or 7 minutes: in the sheep this is from 1 in 2000–10,000, in the cat 1 in 6000–25,000, in the human 1 in 20,000–60,000 and in the rabbit 1 in 50,000–120,000. Of these four animals the cat has the most satisfactory cells for haemolytic experiments, as the suspension, in this dilution, is of a beautiful rich colour and the end points are sharp; the rabbit is the least satisfactory on this score, and, owing to the greater liability of clotting, the blood is not so easy to obtain. As the p_H has a definite effect on haemolysis the solutions were tested with regard to this, but as will be seen from Table II the variations were small and quite unconnected with the results. It is obvious too that the effect on haemolysis in these systems is not connected with the molecular weight, and indeed it is not justifiable in the present state of knowledge to seek correlations between the action of the ions and their other physical properties. It is interesting to note, although no reason is assignable at the moment, that lithium is acceleratory in the sheep and inhibitory in the other cases, whilst calcium is inhibitory in the rabbit and acceleratory in the others. Two figures are given for the cat and the human showing c_1 and c_2 plotted against each other for the various salt systems. It will be seen at a glance that the action of the kations is very different. Similar plottings of the sheep and rabbit merely emphasise this still further.

These results are so much at variance with those of Höber [1917], and Höber and Nast [1914], that some discussion of these papers is requisite.

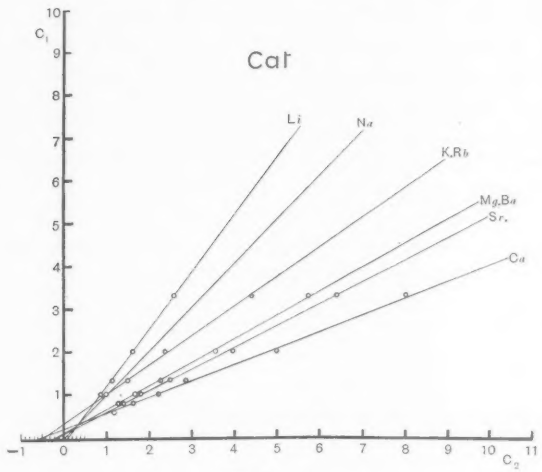

Fig. 1.

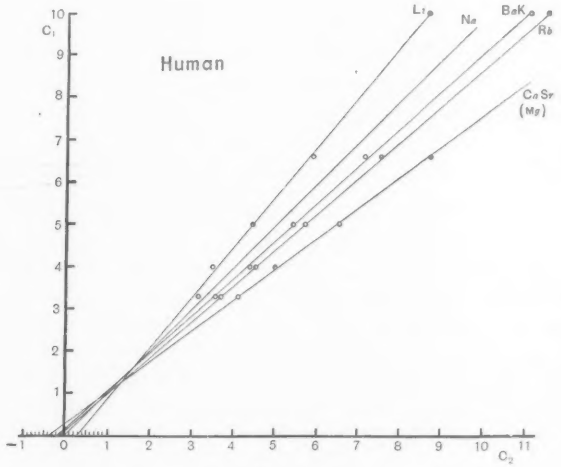

Fig. 2.

Note: the line of Mg is very near to, but not actually coincident with, that of Ca and Sr.

In the first case it may be pointed out that their methods contain certain fallacies which obscure the results. It can be readily calculated from Höber's own figures that during his experiments with saponin the cells were suspended in a medium containing serum in a concentration of not less than 1 in 600, and such a concentration in itself will produce a very considerable inhibition of haemolysis, as may be seen from the tables of Ponder [1925]. Thus it follows

that the kinetics of the haemolytic systems used were such as to be incapable of simple solution even if the exact concentration of serum were known, which was not the case. The experiments were conducted in an ice-chest, and whilst this is quite a permissible method it has the objection that it places inconvenience and experimental error at a maximum. Especially, it so lengthens the end point that no accurate time relation can be ascertained. This, however, was not attempted as the amount of haemolysis is throughout expressed in such vague terms as "weak," or "strong haemolysis," "colourless" and the like. These pseudo-quantitative measurements are of such a nature that the conclusions drawn from them are quite unreliable: even then the results are tabulated in a way which permits of considerable variety of interpretation.

Among their findings they state that variations of the saponin concentration result in a reversal of the order of ionic activity. This would imply that the time dilution curves crossed, a result which has not been met with throughout the long series of different experiments conducted by Ponder's method.

The results recorded in this paper are quite opposed to Höber's, and indeed often diametrically opposite, but they are given additional weight by the mathematical analysis which results in all cases in a straight line relationship between the curves, in which incidentally, each point is checked by that above and below it. These straight line relationships are found in the cases of many other systems, such as those containing sugars [Ponder and Kennedy, 1926], certain acids and alkalis, and other acceleratory and inhibitory substances.

Summary.

The effect of hypotonicity on a cell-saponin-NaCl haemolytic system is shown to be inhibitory.

The effects of various kations in cell-saponin-salt systems are compared with a standard cell-saponin-NaCl system, and with each other. The method employed measures the resistances of the systems in terms of a constant which can readily be evaluated.

The equations applicable to these systems are identical with the general equations for inhibition and acceleration. Höber and Nast's results are not confirmed by these methods.

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XXXII. THE HEAT-STABLE PEROXIDASE OF BACTERIA.

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Report to the Medical Research Council.

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The term peroxidase was originally applied to an enzyme of the higher plants that was capable of blueing guaiacum in the presence of hydrogen peroxide. Plant extracts that had been boiled gave no peroxidase reaction. Later work showed that certain iron-containing substances, such as haemoglobin, also gave the peroxidase reaction. Buckmaster found that these iron-containing substances gave the reaction equally well after they had been boiled, and he used the term pseudoperoxidase for this reaction [Buckmaster, 1908]. There are certain indications in the literature that bacterial peroxidase is also heat-stable, and the present investigation confirms this observation. In this respect therefore the peroxidase of bacteria resembles pseudoperoxidase, and differs from the peroxidase of the higher plants, which is destroyed by heat.

Detection of peroxidase. The following reagents were used for the detection of peroxidase in bacteria:

(i) 1 % solution of benzidine in 50 % alcohol;

(ii) 1 % solution of guaiacum in 95 % alcohol. The guaiacum was obtained from the inside of a lump of resin, boiled with alcohol in the presence of animal charcoal to remove peroxides, and then filtered.

In each case, two drops of the freshly prepared solution were mixed with the material to be tested in a white dish, and two drops of hydrogen peroxide (20 volumes) added. In both tests a blue colour indicates the presence of peroxidase.

Pure cultures of various aerobic bacteria, obtained from the National Collection of Type Cultures, were grown in Roux bottles of tryptic broth. Good growth of the anaerobe *B. sporogenes* was obtained as follows: 6-inch test-tubes, half full of glucose tryptic broth, were autoclaved, cooled rapidly, and inoculated at once with material from a similar glucose broth culture. Cultures were incubated at 37° for 18 hours, and then centrifugalised. The clear broth was decanted off, and the bacteria were ground up in a mortar with distilled water to wash away traces of broth. The emulsion was again

centrifugalised and the water decanted off. This process was repeated, and the bacterial mass was finally emulsified with enough distilled water to give a very thick suspension, which was tested for the presence of peroxidase. It was found that benzidine and guaiacum gave similar results. In all cases controls were carried out using the reagents without addition of bacteria. The guaiacum + H_2O_2 control gave a very slight blue colour when left for several hours, but the benzidine + H_2O_2 control gave no trace of blue colour. Another advantage of benzidine was that it generally gave a deeper blue colour with bacterial peroxidase than was obtained when guaiacum was used.

The following bacteria were investigated: *B. faecalis alcaligenes*, *B. pyocyaneus*, *B. fluorescens*, *B. prodigiosus*, *B. proteus vulgaris*, *B. coli*, *B. paratyphosus* A, *B. paratyphosus* B, *B. subtilis*, *B. megatherium*, *Staphylococcus aureus*, *Streptococcus acidi lactici*, and the anaerobe *B. sporogenes* (Metchnikoff). All these bacteria were found to give the peroxidase reaction, although in the case of the *Streptococcus* and *B. sporogenes* the blue colour was very much less intense than with the other bacteria investigated. This is another example of the way in which *S. acidi lactici* differs from most aerobes and resembles the anaerobes [see Callow, 1923, 1924]. In all cases the peroxidase was stable to heat and, with the exception of the two organisms just mentioned, a very intense blue colour was given by bacteria that had been boiled in a Pyrex flask for an hour, cooled, and tested with benzidine + H_2O_2. The peroxidase was found to be remarkably stable, as shown by the following experiment. *B. faecalis alcaligenes* was grown in bulk, washed, and dried in a vacuum desiccator over sulphuric acid. The resulting dry powder was ground up with distilled water, the washings being removed after centrifugalisation. After being washed seven times, the bacterial mass was suspended in distilled water and boiled for 5 minutes. The water was removed as before, and the bacteria finally treated with 98 % alcohol and again dried. The resulting "thermostable residue" [see Hopkins and Dixon, 1922] gave the peroxidase reaction, as well as being positive to the nitroprusside test. This does not of course imply that the fixed SH group is responsible for the peroxidase reaction, and indeed this is highly improbable, because certain substances such as boiled egg-white which contain a fixed SH group give no peroxidase reaction.

In most bacteria the blue colour obtained with benzidine + H_2O_2 is actually very much deeper after the bacterial emulsion has been boiled than it was before. Sometimes, in fact, practically no blue colour is obtained with the fresh organism. Generally, with fresh bacteria, a blue colour appears which fades away after about half an hour, unlike the permanent blue colour that is obtained with boiled bacteria. Fresh, washed cultures of *B. faecalis alcaligenes*, etc., are also able to cause fading in the blue colour obtained with a weak solution of haemoglobin + benzidine and H_2O_2. Most bacteria contain large quantities of catalase, which may possibly interfere with the peroxidase reaction by decomposing the H_2O_2. But there appears to be some other factor in fresh bacteria that affects the peroxidase reaction because, although catalase

might possibly prevent the formation of the blue colour, there is no reason to suppose that it can destroy the blue colour that has already been formed in the peroxidase reaction. Moreover, the factor that causes the blue colour to fade appears to be less stable than catalase, and tends to disappear when emulsions of bacteria are kept, or dried, etc. The action of heat is shown by the following experiment. A suspension of fresh, washed *B. faecalis alcaligenes* gave with benzidine + H_2O_2 a very slight blue colour which faded completely in 10 minutes. After being kept at 83° for 1 minute, it gave a deep blue colour that was quite permanent.

It was found that the blue colour obtained in the peroxidase reaction could be removed by the action of certain reducing agents, such as the SH group (in neutralised thioglycollic acid). It appeared possible, therefore, that some heat-labile reducing system in bacteria might be responsible for the fading of the blue colour. The bacteria that were used for examining the fading of the blue colour were: *B. faecalis alcaligenes*, *B. pyocyaneus*, *B. fluorescens*, *B. prodigiosus*, *B. proteus*, *B. subtilis*, and *Staphylococcus aureus*. Fresh young cultures of all these bacteria gave a rapidly fading blue colour with the peroxidase reagents, and in each case a very deep permanent blue colour was obtained after the organism had been boiled. It may be mentioned that the factor that causes fading was found in *S. acidi lactici*; fresh, washed material gave either a very slight fading blue, or no blue colour at all, but after boiling a distinct, although slight, permanent blue colour was obtained. Two reducing systems of bacteria were investigated to see whether they corresponded with the factor that causes the blue peroxidase colour to fade.

I. *Reduction of nitrates.* A most striking fact in connection with the peroxidase reaction of fresh bacteria, such as *B. faecalis alcaligenes*, etc., is that the fading of the blue colour does not take place if nitrates are present. This suggested that possibly there might be some connection between the removal of the blue colour and the reduction of nitrates by bacteria. Young cultures of bacteria were washed in the usual way, and mixed with distilled water to form strong, even emulsions. To 3 cc. of bacterial emulsion, 1 cc. of a 5 % solution of potassium nitrate was added. The mixture was placed in a water-bath kept at 60° for 3 minutes. It was then cooled, and tested for nitrites by two methods.

(1) Addition of potassium iodide, starch paste and sulphuric acid, which gives a blue colour with nitrites.

(2) The Griess-Ilosvay reagent, which gives a pink colour with traces of nitrites.

Certain bacteria were investigated in this way, and it was found, for instance, that *B. pyocyaneus*, *B. fluorescens*, *B. coli*, and *Staphylococcus aureus* reduced nitrates to nitrites under these conditions, but boiled suspensions of these bacteria were inactive. *B. faecalis alcaligenes* gave no trace of nitrite formation. In order to exclude the possibility of obtaining negative results by not taking enough material, a measure of the strength of suspension was

obtained as follows: 1 cc. was placed in a small tared centrifuge tube and centrifugalised. The water was decanted off, and the tube containing the bacteria was dried in a vacuum desiccator over sulphuric acid, and weighed. It was found that suspensions which contained 0·03 g. dry weight of bacteria per cc. gave a strong nitrate reduction with the bacteria mentioned above, but that even with 0·06 g. per cc. *B. faecalis alcaligenes* showed no trace of nitrite formation [cf. Quastel, Stephenson and Whetham, 1925; Quastel and Wooldridge, 1925].

II. *Reduction of methylene blue.* The reduction of methylene blue was studied in a way similar to that used for nitrates. A solution of methylene blue was added to washed suspensions of bacteria, which were then placed in a water-bath at 60°. The surface of the mixture was protected by a layer of olive oil to prevent re-oxidation of the leuco-methylene blue by air. The time taken for known suspensions of bacteria to reduce various concentrations of methylene blue was observed. Great difficulty was experienced in this part of the investigation. Since the reducing system was heat-labile, an attempt was made to obtain emulsions of bacteria that would reduce given solutions of methylene blue when kept for only 2 or 3 minutes at 60°. Unfortunately, strong emulsions of bacteria were capable of reducing only very weak solutions of methylene blue in such a short time. Finally, 0·1 cc. of *M*/5000 methylene blue was added to the bacterial suspension. The strength of the suspension was found as before, and the time taken for complete reduction of the methylene blue was noted. The following results are typical:

Organism	Number of washings	Approx. dry weight in g. of bacteria in suspension	Time in seconds for reduction of methylene blue solution
1. *B. faecalis alcaligenes*	2	0·023	90
2. *B. pyocyaneus*	2	0·030	80
3. *B. fluorescens*	2	0·016	100
4. *B. coli*	1	0·055	120
5. *B. megatherium*	1	0·037	100

Boiled emulsions of all these bacteria had no reducing power. The general conclusion from the results is that the bacteria which were found to reduce methylene blue, under the given conditions, all gave a fading blue colour with the peroxidase reagents. Further evidence on this point was obtained by observing the effect on the peroxidase reaction of certain substances which are known to affect the system in bacteria that is responsible for the reduction of methylene blue. The following substances were selected:

(1) *Neutral sodium formate.* This has been shown to accelerate the reduction of methylene blue by *B. coli*, *B. faecalis alcaligenes*, and *B. prodigiosus* [Quastel and Whetham, 1925; Quastel and Wooldridge, 1925].

(2) *Propyl alcohol and cyclohexanol.* These substances have been shown to retard reduction of methylene blue by *B. coli*, a small quantity of cyclohexanol producing complete inhibition [Quastel and Whetham, 1925].

The following experiment shows that the fading of the blue peroxidase colour is also accelerated by sodium formate, and retarded by propyl alcohol and cyclohexanol. A fresh, young culture of *B. faecalis alcaligenes* was washed and emulsified in water. Half of the emulsion was boiled for 5 minutes, and cooled. The table shows the substances added, and their effect on the fading of the blue peroxidase colour. In each case 1 cc. of bacterial suspension was used.

Condition of suspension	Substance added	Colour obtained on addition of benzidine and H_2O_2
1. Fresh	None	Slight blue which faded rapidly
2. Boiled	None	Deep permanent blue
3. Fresh	Neutralised sodium formate	No blue colour
4. Boiled	Neutralised sodium formate	Deep permanent blue
5. Fresh	2 drops of propyl alcohol	Deep permanent blue
6. Boiled	2 drops of propyl alcohol	Deep permanent blue
7. Fresh	2 drops of cyclohexanol	Deep permanent blue
8. Boiled	2 drops of cyclohexanol	Deep permanent blue

Cyclohexanol was added to No. 3, but still no blue colour appeared. This suggests that, as found by Quastel and Whetham with the methylene blue reducing system, cyclohexanol acts not as an oxidising agent, but as an inhibitor of the reducing system. The above eight experiments were repeated with *B. coli*, and similar results were obtained.

The function of peroxidase. Keilin's recent work [1925] is of great interest in connection with the function of the heat-stable peroxidase. He has found that the respiratory pigment cytochrome is common to animals, bacteria, yeast, and the higher plants, and he considers that "cytochrome and its derivatives are responsible, at least partly, for the peroxidase reaction in organisms." Moreover, he has shown that the aerobic bacteria which give a strong peroxidase reaction contain cytochrome, whereas *S. acidi lactici* and *B. sporogenes*, which give only a very slight peroxidase reaction, contain no cytochrome.

Summary.

1. A strong peroxidase reaction was given by *B. faecalis alcaligenes*, *B. pyocyaneus*, *B. fluorescens*, *B. prodigiosus*, *B. proteus*, *B. coli*, *B. paratyphosus* A, *B. paratyphosus* B, *B. subtilis*, *B. megatherium* and *Staphylococcus aureus*.

2. *S. acidi lactici* and the anaerobe *B. sporogenes* gave only a very slight peroxidase reaction.

3. In all cases the peroxidase was stable to heat.

4. With fresh, young, washed cultures of many bacteria, the blue colour formed in the peroxidase reaction faded rapidly. After being boiled, all the bacteria examined gave a permanent blue colour.

5. The heat-labile factor responsible for the fading of the blue peroxidase colour showed certain points of resemblance to the methylene blue reducing system.

In conclusion, I gladly take this opportunity of thanking Sir F. G. Hopkins for the interest he has taken in this work.

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XXXIII. THE CONCENTRATION OF DIASTASE IN THE URINE THROUGHOUT THE DAY.

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(*Received February 10th, 1926.*)

The investigation forming the subject of this paper was a continuation of an enquiry into the origin and method of excretion of urinary diastase. The concentration of this enzyme in the urine during the various parts of the day, and the effect of meals over an observation period of 24 hours are specially dealt with.

In a previous paper [Cohen, 1925] it was shown that whereas the concentration of diastase in the blood remained remarkably constant over various periods of observation, that in the urine showed marked variations throughout the same periods. Following the intake of food there appeared a definite, though at times quite small, increase in the amount of diastase in the urine, but, as in some cases almost equivalent changes in concentration took place during starvation experiments, some doubt was expressed as to whether the taking of food was really the cause of this increase in concentration of the enzyme in the urine. Arising out of these facts it was obvious that the examination of isolated samples of urine for clinical purposes would tend to yield very unreliable information. For further research it became desirable to know the conditions under which one could collect a specimen of urine which would yield the most reliable results as a basis for judging deviations from the normal. In the course of some more experiments on the effect of meals on the output of urinary diastase, it was found that the response to meals appeared greatest after the first meal in the day. This is in agreement with Stocks [1916], who from the results of seven cases concluded that the concentration of amylase in the urine is at a maximum just after breakfast. This is as one might expect it to be, for it appears reasonable to suppose that after a night's rest the digestive system would respond more readily to stimulation by food than at some time during the day after meals have been taken. In the same way it was thought that the nightly rate of excretion of diastase, in view of the absence of complications due to meals, might give fairly constant results from night to night, and so this point was investigated.

The output of diastase during the night was obtained from experiments carried out on the following lines. The subject was instructed to empty the

bladder on retiring for the night, to discard this specimen, and to note the time. On rising in the morning the bladder was again emptied, the urine collected, and the time again taken. The volume of urine passed was measured, the diastatic index of the specimen was estimated, and from the data so obtained the rate of excreting units of diastase per hour was calculated. It was soon noticed that the same individual showed great variations in the rate of output of enzyme on different nights. For instance, in one case the same person showed during one night an excretion rate of 2508 units diastase per hour, and 15 days later a rate of only 350 units per hour. To obtain further information a consecutive series of nightly urines was collected, the same conditions being observed each night according to the following procedure, and the diastatic rate was determined. The method used for the latter in these experiments was the one described by Wohlgemuth [1908], and modified by Dodds [1922].

The individual on whom the experiment was carried out adhered to the following routine throughout the period of observation. Dinner was taken each day at about 7 p.m., and no more food or drink taken till breakfast the following morning. At 11 p.m. the bladder was emptied and this specimen was discarded. The interval of 4 hours between the meal and the commencement of observation was allowed in order to overcome as far as possible any effect the meal would have upon the urinary diastase. At 7.30 a.m. the next morning the night's urine was collected, its volume was measured and the diastatic rate calculated. It was seen after a few days that the results obtained varied markedly from night to night. The nightly rates of diastase output not yielding constant results, it immediately suggested itself that the most likely specimen of urine to show a constant diastatic concentration from day to day, would possibly be the one collected just before the breakfast meal. The advantage in this case was that this specimen was farthest away from the last meal and, moreover, it was taken after a night's rest. From the 8th day of investigation, therefore, it was decided in addition to the nightly urine to collect and estimate each day the half-hourly specimen from 7.30 to 8 a.m. The results so obtained are represented graphically in Fig. 1 along with the nightly ones. The observations were continued for a period of more than 2 months. Inspection of the curves will show at a glance the marked variations in both series of figures, which, however, rise and fall together throughout the period of observation. It will also be seen that the rate first thing in the morning is higher than the nightly one. On the 50th day there occurred in the half-hourly specimen an unexpected enormous rise in diastatic concentration although experimental conditions were unaltered. Similar unaccountable increases in a single half-hourly specimen will be referred to later. During the last 17 days of the experiment this same subject collected 24-hourly specimens of urine. The daily urine from 8 a.m. to 11 p.m. was collected and, with the other specimens collected as before, it was possible to deduce the average rate of excretion of diastase into the urine throughout the whole day.

These figures, together with the total 24-hourly output, are given in the following table, which also serves to demonstrate the manner in which the results were calculated in all these experiments.

Excretion of urinary diastase over periods of 24 hours.

Day of experiment	Volume cc.		Units diastase per cc.	Units diastase per hour	Units diastase 24 hours
	Total	Per hour			
47	1082	45	33	1485	35,706
48	1222	51	25	1275	30,550
49	1528	64	25	1600	38,200
50	1527	64	25	1600	38,175
51	1433	59	25	1475	35,825
52	1341	56	22	1232	29,502
53	1402	58	25	1450	35,050
54	1398	54	25	1350	34,950
55	1389	58	29	1682	40,281
56	1488	62	25	1550	37,200
57	1197	50	29	1450	34,713
58	1308	55	25	1375	32,700
59	1492	62	25	1550	37,300
60	2356	98	20	1960	47,120
61	1228	51	25	1275	30,700
62	890	37	29	1073	25,810
63	1368	57	33	1881	45,144

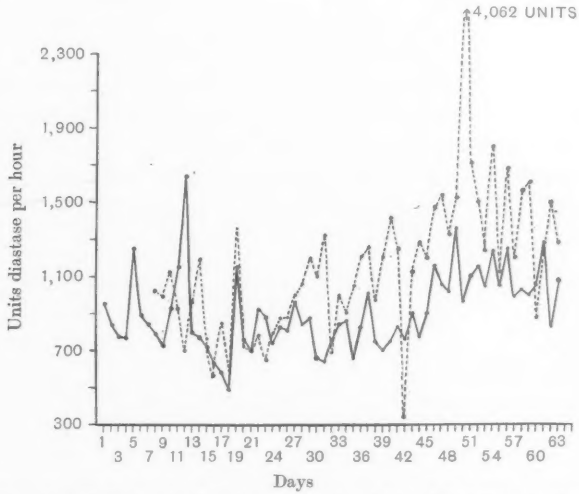

Fig. 1. Excretion of diastase in the urine during the night and first thing in the morning.
- - - - - - Half-hour specimen. ———— Night specimen.

From the figures in this table in conjunction with the curves in Fig. 1, one is in a position to compare the rates of excretion of diastase into the urine during the whole cycle of 24 hours, first thing in the morning and during the night. The curves in Fig. 1 show that the half-hourly specimen has a higher rate than the average rate during the night, a finding which is contrary to what one would expect. On the other hand, the average rate throughout the

whole 24 hours is higher than the average nightly rate alone, it is clear therefore that the rate during the daytime alone must be still higher than the nightly one. That is to say, during the food-taking period diastase is being excreted into the urine at a greater rate than during the time when the digestive organs are at rest, but it cannot be inferred from this that it is the partaking of food that has produced this effect.

The last column in the table shows that the total output of diastase each day was between 25,000 and 47,000 units. Based on theoretical considerations, *i.e.* taking 1500 cc. as the average amount of urine passed by an adult in 24 hours, and 10 to 30 units, on which most authorities agree, as the average diastatic index, one obtains a quantity of enzyme passed in 24 hours varying from 15,000 to 45,000 units. Harrison and Lawrence [1923] give the normal range as lying between 8000 and 30,000 units. In examining 24 hourly urines of patients other than nephritic and pancreatic cases, who are confined to bed in the wards, figures as low as 6000 units have been found. Patients on restricted diet also give low figures. Now it will be seen here, as has also been pointed out in previous work, that the approximate readings given by the method employed are partly responsible for the irregular curves in the table. For clinical purposes this standard method of Wohlgemuth's is still of great value, for only very high or very low figures are considered, but for research purposes a method is required which will give readings at least to the nearest whole unit. Such a method was described in conjunction with Dodds [Cohen and Dodds, 1924, 1]. Using this method, an investigation was carried out with reference to the excretion of diastase into the urine over a continuous period of 24 hours. Half-hourly specimens were taken from feeding and starving individuals, so as to observe the effect of meals. Hitherto the experiments had been carried out over much shorter periods and never during the night.

Five healthy men working in the Biochemical Department were selected as subjects, three of whom took food at the usual times and two of whom acted as starvation controls. The latter also refrained from drinking throughout the period of the experiment. Sleep was, of necessity, an impossibility. Any results obtained from these experiments are not without somewhat serious objection. Thus it can be argued that the subjects were being examined under pathological conditions, since no sleep was obtained. It is suggested that conclusions based on half-hourly specimens of urine are of greater value than any observations on the whole of the night's urine obtained when the subject wakes in the morning.

The subjects dined at 7 p.m. and the experiment was commenced at 9 p.m. The subjects emptied their bladders at the commencement of the observation and this specimen was discarded. At half-hourly intervals throughout the following 24 hours specimens of urine were collected. The volume of each specimen was measured as soon as passed, and a few drops of toluene were added in order to preserve the specimen. Estimations of the diastatic values

were begun immediately during the hours of the experiment, and continued on the following days till the 240 urines had been estimated[1]. The results were recorded graphically as rates of passing units of diastase per hour. Two of these curves, one from an individual who took food at the usual meal times, and the other from an individual who fasted, are represented for the purpose of comparison in Fig. 2.

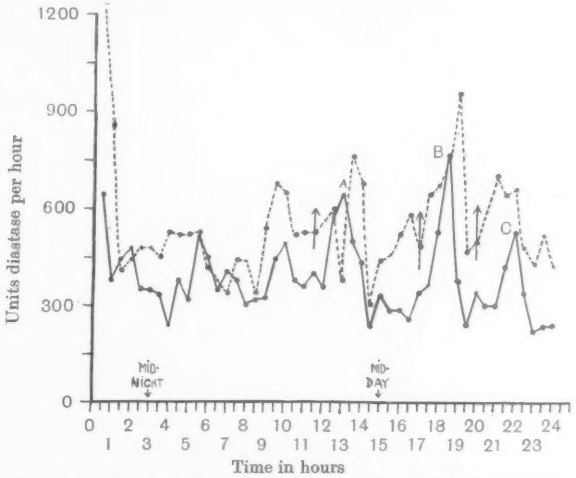

Fig. 2. Excretion of diastase in the urine over a period of 24 hours.
– – – – – – Normal. Arrows indicate meals. ———— Starving.

From the previous observations [Cohen, 1925], on meal and starvation effects over short periods, it was noticed that, firstly, there was a definite rise in diastatic output within $\frac{1}{2}$ to 1 hour following a meal, and secondly, during the correspondingly short starvation controls the amplitudes of the curves were not diminished, which might have been the case had the variations in the figures been due solely to the intake of food. On inspecting Fig. 2 it will be seen that after each meal, which is represented by an arrow in the normal curve, there is a rise in diastase output. The starvation curve should supply information as to whether these rises are really due to meals, for it would be expected that the effect of not taking food would produce a much smoother curve. Three marked rises are seen culminating at the points *A*, *B* and *C*. These rises are quite as large in amplitude as the rises after meals in the normal curve. It is remarkable that these points correspond approximately in time to what appears to be an effect due to meals in the normal curve. It must therefore be concluded that the increase in diastase output after a meal is not really due to the intake of food, but is a coincident rise which would probably have occurred had no meal been taken.

[1] Several other constituents, such as urea, total nitrogen, etc., were determined on these urines and the findings were recorded elsewhere [Cohen and Dodds, 1924, 2].

It has previously been recorded that in an experiment one half-hourly specimen without apparent cause showed an enormous increase in diastatic concentration. Two further such observations appeared in these experiments. One specimen showed an increase in rate to about 4000 units, the one preceding it being about 500 units and the one following about 700 units, and another increased from about 900 to 3000 units. These extraordinary figures are however not subsequently maintained, and have only a slight effect on the average rate taken over several hours. Such facts point out the danger of working with isolated specimens.

SUMMARY.

The rate of appearance of diastase in the urine is greater during the daytime than at night. This higher rate during the period of meals is not due to the taking of food. Variations occur from day to day in the total daily and nightly output of diastase in healthy individuals. It is therefore clear that no significance can be placed on figures unless they differ markedly from the normal over a period of several hours.

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XXXIV. THE GLUCOSE IN BLOOD.

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(*Received February 20th, 1926.*)

Differences which have been stated to exist in the forms of glucose in the blood in health and in disease have focussed attention on the relation between amounts of sugar obtained in the same sample of blood by various quantitative methods. The older literature on the subject is reviewed by Bang [1913] and by Stepp [1922] and need not be gone into here.

Up to the present, except for the difficult polarimetric estimation, most of the methods have relied on the reducing power of the deproteinised blood. The results, however, are not concordant and depend partly on the conditions of oxidation. Thus Benedict states [1925] that his picric acid method, devised with Lewis [1915], gives considerably higher results than the Folin-Wu [1920] method and is confident that the values so obtained indicate more than the actual sugar content of the blood.

Grafe and Sorgenfrei [1924] were impressed by an unsatisfactory situation in which all estimations of glucose in blood were variants of reduction methods and suggested as a control the use of the single Barcroft manometer using yeast to ferment the blood. They found that in normal cases the reduction method of Benedict gave substantially the same results as the fermentation method. In cases of diabetes, however, the differences were very great. The fermentation values were lower than the reduction values by as much as 40 %. The statements were of such interest that we decided to go into them more closely and to repeat them.

An analysis of the tables of Grafe and Sorgenfrei show for non-diabetics a ratio of fermentable substance to reducing substance $\left(\frac{100F}{R}\right)$ of from 73 to 103. In diabetics the ratio varies from 61–102. In a set of analyses of bloods after food and insulin, ratios ranging from 53–107 were obtained. So far as we were able to make out, no definite regularity or difference between normal and diabetic bloods could be observed.

Whilst it is recognised that there may be fermentable substances in the blood other than glucose, it would seem that the high values obtained in some instances by Grafe and Sorgenfrei were worthy of further study, especially in view of the fact that the reduction method employed by these workers was one known to give high results. During the course of the present work there appeared a paper by Benedict [1925] in which he describes a new and more

selective method for the estimation of blood sugar. This method, he believes, gives results more nearly approaching the true content of glucose in the blood than any other at present available. As a result of his work he came to the conclusion that the true sugar content of the blood is probably only 60–75 % of that which is now stated to be present by current reduction methods. By using the new Benedict method as a control of our fermentation results, we were in a position to see whether the 25–40 % difference found by Benedict corresponded with any discrepancy found by fermentation.

The method used by us was one partially described in a previous paper [1925]. The blood was diluted with equal parts of distilled water and 1·0 % orthophosphoric acid and 3·0 cc. of the mixture taken for fermentation, so that 1·0 cc. of blood was actually fermented.

After dilution of the blood it was very vigorously shaken to expel carbon dioxide and to ensure complete aeration. In some experiments with animal bloods additional estimations were made of a control blood to which a definite amount of glucose had been added. We were thus able to obtain some idea of the fermentative quality of clean yeast on glucose in the presence of blood. We had previously ascertained that it was not satisfactory to obtain the information from the action of yeast on an aqueous solution of pure glucose alone. The addition of blood accelerates the fermentation. In a special column is given the percentage of added glucose fermented.

The routine method for the determination of sugar in blood employed in this laboratory is that of Hagedorn and Jensen [1923] which gives in our hands, with amounts of sugar added to blood, nearly the theoretical figure. Höst and Hatlehol [1920], in their comparison of four methods, give figures which show the method to give an average error of 3·2 % and the greatest error of 9 %. An inspection of their tables seems to show that the error is one giving less glucose than that added to the blood. In this our results confirm those of the Danish workers. The standard glucose solutions used by us were made in the beginning from a sample for which we are indebted to the Carbohydrate Laboratory of the U.S. Department of Agriculture. It was stated to be over 99·0 % pure glucose. It was dried in a thin layer in a vacuum desiccator for 24 hours over sulphuric acid before being weighed. In some of the later work a highly purified commercial glucose was used. A comparison of this with the former sugar showed no appreciable difference. For the Benedict estimation, the standard sugar solution was preserved with toluene. For fermentation, the sugar was made up frequently in 0·1 *N* sulphuric acid and just before use each day was neutralised with the requisite amount of 0·1 *N* sodium hydroxide and diluted to 0·1 %.

The comparisons are divided into (I) animal bloods, (II) non-diabetic human bloods, (III) diabetic bloods. The number of samples which we have examined is large and only a selection of those comparisons are given which were done after the paper of Benedict appeared. They represent as faithfully as possible the general result of the investigation.

I. *Animal Bloods.*

	Blood sugar %					% of added glucose fermented
	Hagedorn and Jensen	Fermentation	Benedict	$\frac{100F}{\text{H. and J.}}$	$\frac{100B}{\text{H. and J.}}$	
Sheep	0·058	0·038	0·064	65	110	—
,, ...	0·055	0·031	0·044	56	81	—
,, ...	0·050	0·033	0·048	66	96	91
,, ...	0·040	0·033	0·049	82	122	86
Bullock	0·064	0·044	0·062	68	97	—
,, ...	0·150	0·098	0·156	65	103	99
,, ...	0·162	0·123	0·149	76	92	77
Pig	0·079	0·071	0·065	90	82	—
,, ...	0·086	0·048	0·072	55	83	—
Dog	0·086	0·048	0·073	56	85	99
Cat	0·250	0·220	0·276	88	110	—
Rabbit	0·034	0·018	0·027	53	79	—

II. *Non-diabetic Bloods.*

Nephritis	70 mg. urea %	0·078	0·062	0·085	39	110	—
Normal		0·086	0·051	0·100	59	115	—
,, ...		0·100	0·051	0·116	51	116	—
Nephritis	68 ,, ,,	0·086	0·043	0·080	50	94	—
,,	(?) 30 ,, ,,	0·074	0·035	0·076	47	104	—
Cystitis	40 ,, ,,	0·100	0·050	0·094	50	94	—
Prostate	159 ,, ,,	0·094	0·040	0·099	42	105	—

III. *Diabetic Bloods.*

0·110	0·053	0·102	48	92	—
0·220	0·138	0·210	63	96	—
0·144	0·058	0·162	40	112	—
0·188	0·106	0·177	56	94	—
0·152	0·104	0·148	68	97	—
0·140	0·074	0·106	52	76	—
0·240	0·204	0·287	85	119	—
0·252	0·161	0·214	64	85	—

An examination of the tables may be divided into two parts. (1) The concordance between the new Benedict method and that of Hagedorn and Jensen. With animal bloods the ratio $\frac{100B}{\text{H. and J.}}$ ranges from 74 to 122. There is no obvious connection between sugar level or class of animal dealt with. With non-diabetics the balance, if any, is in favour of showing that the Benedict method gives higher values. With diabetics the Benedict figures are, perhaps, somewhat lower than those of Hagedorn and Jensen, but here again one obtains high figures; for example, a case with a hyperglycaemia of 0·24 % giving a $\frac{100B}{\text{H. and J}}$ ratio of 119.

(2) Turning now to the ratios between the Hagedorn and Jensen values and the amount of glucose found by fermentation, we find in all cases lower figures from fermentation than are found when we obtain our results by a reduction method. The ratios vary from 39 % to 88 %. It is interesting to note that these high percentages are associated with a high total blood sugar, a result which one might anticipate if one assumes that the hyperglycaemia is the principal feature in the change in metabolism. Here again is an exception in the last case among the diabetic bloods.

The average level of $\frac{100F}{\text{H. and J.}}$ in non-diabetics is close to 50, when none of the blood sugar percentages exceeds 0·1%. That the low ratios are not due to incomplete fermentation is shown by the comparisons with the fermentation of added glucose. The values vary from 77 to 99 %. The average of all determinations is 90 %. Taking this as the correct figure, one finds that the discrepancy between the reduction and fermentation method is as high as 50 % in some cases of low total reducing substance in the blood and on an average between 40 and 30 %. One may safely set down these latter figures as those which represent the proportion of the total reducing substances of the blood which are not glucose. These are in agreement with the conclusion reached by Benedict himself. The variations are, however, large and we have not been able to make out any rule in the matter.

For part of the expenses of this work we are indebted to the Medical Research Council.

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XXXV. THE ESTIMATION OF GLUCOSE IN BIOLOGICAL MATERIAL.

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(*Received February 19th, 1926.*)

The methods of estimation of glucose in general use in biological research are all based on its power of reducing various substances in alkaline solution. Amongst others the following compounds have been employed: copper salts, sodium picrate, potassium ferricyanide, methylene blue, indigotetrasulphonic acid and dinitrosalicylic acid. Additional variations have been introduced by different methods of estimating the amount of substance reduced and by different compositions of the various solutions used.

Recent years have witnessed the development of methods of estimation of glucose which are applicable to very small amounts or to a particular medium, *e.g.* blood or urine.

Two tests have usually been employed in order to determine the fitness of a given method for a given piece of physiological research. Firstly, a demonstration that certain other biological substances whose presence is suspected, *e.g.* acetone or β hydroxybutyric acid, do not, in a pure aqueous solution, reduce the substance used to oxidise the glucose. Secondly, the correct recovery of known amounts of glucose added to the biological material for which the method is devised. If these criteria are satisfied, the original reducing power of the material is calculated as, and attributed to, its glucose content. The standard of reference employed is usually a solution containing solely a known weight of the purest glucose obtainable.

For clinical purposes any standard method probably gives reliable and valuable information when used by an experienced worker. For research work, however, it would seem highly desirable to gain some knowledge that would either confirm or modify the above assumption in a quantitative manner.

Comparative research on different methods of estimation of glucose when applied to the same biological fluid has not hitherto yielded very encouraging results.

De Wesselow [1919] found that the Lewis-Benedict picric acid method yielded results 30–50 % higher than those obtained by MacLean's method, due in the main to unknown substances in the corpuscles. Folin and Wu [1919] stated that their method yielded lower results than the Lewis-Benedict

method. Höst and Hatlehol [1920] compared four methods over a large number of normal bloods and found that Bang's [1918] copper method and that of Hagedorn and Jensen [1923] agreed fairly well, as did Folin and Wu's method with the Myers-Bailey [1916] modification of the Lewis-Benedict method, but that the two latter frequently yielded decidedly higher values than did the two former. They add: "It is uncertain whether any of the blood-sugar methods hitherto used give only the amount of glucose." The fact [Wishart, 1920; Macleod, 1921] that direct determinations of the amounts of glucose present in whole blood, plasma, and corpuscles, do not tally with the haematocrit determinations is, in reality, a serious criticism against the validity of the methods employed, because it implies that whatever be the nature of the various reducing agents present, the effect of each depends in some degree on the varying concentrations of the others or possibly of some of the non-reducing substances present. The discovery of insulin and the subsequent researches on hypoglycaemia have brought the question of glucose estimation into prominence.

Winter and Smith [1924] stated that in hypoglycaemic convulsions following an injection of insulin the blood of rabbits is frequently practically free from glucose although their blood sugar, as estimated by Bang's method, is about 0·04 %. The writer has not been able to observe so complete an absence of glucose from the blood but, as will be shown below, the concentration of glucose under such circumstances may be very considerably below the amount estimated to be present by certain micro-methods. According to Macleod [1924] the level of blood sugar at which convulsions occur depends on the previous feeding and the quantity of insulin injected.

The problem of the nature, concentration and physiological behaviour of the reducing substances in blood other than glucose is probably of considerable importance.

The absence of close agreement between the standard methods for estimating glucose in biological fluids suggests perhaps that the proportion of other reducing substances to glucose in blood may be by no means insignificant, and that their concentration may vary considerably in different physiological or pathological conditions. It is by no means certain that the same group of substances is always involved.

As a preliminary study, an investigation was made into one aspect of the problem of glucose estimation which has been practically entirely neglected, namely the effect of the presence in the reducing fluid of other biological substances with glucose on the reducing power of the latter.

Taylor [1924] showed that the presence of creatinine or of uric acid interfered with the estimation of glucose by Folin and McEllroy's [1918] direct method.

This method was not a very convenient one for research on such fluids as concentrated blood-filtrates and the effect of such substances as uric acid, in the absence of glucose, cannot be determined.

Accordingly, the two methods chosen for investigation were the Wood-Ost method [Wood and Berry, 1904], modified as to quantities of the reagents by Cole [1920], and the Hagedorn and Jensen method.

The Wood-Ost method has the following advantages.

The degree of alkalinity is less than in those methods using alkaline hydroxides. There is no organic polyhydroxy-substance used. The amount of cuprous oxide is estimated directly after removal by filtration and hence the possible complication of the other biological substances interfering with the estimation of the unreduced cupric salts is avoided. The sole object of consideration was the reducing power of glucose.

The Hagedorn and Jensen method was chosen because of the need for a micro-method. No tartrate or citrate is used in this reagent since potassium ferricyanide replaces the copper sulphate.

Experimental Details.

The experiments consisted for the most part in determining the reducing power by means of the two methods of aqueous solutions containing known amounts of glucose and of the various added substances. The glucose used was a specimen of Kahlbaum's "Dextrose" and its purity was estimated to be 99·6 %. The other organic chemicals were the purest obtainable and were carefully recrystallised.

The concentrations of uric acid and of creatinine employed were admittedly considerable in order to demonstrate their effects as clearly as possible. The concentrations of amino-acids are not unreasonable in comparison with the amount of glucose present. The amino-acid nitrogen of a protein-free blood filtrate varies from 6–10 mg. per 100 cc. of blood. This corresponds to a total concentration of amino-acids of 40–70 mg. per 100 cc. of blood, *i.e.* from one-half to two-thirds of the estimated concentration of reducing carbohydrates.

The following are examples of the results obtained:

A. *Wood-Ost method; values in mg. in the 10 cc. of fluid used.*

	Glucose taken	Uric acid	"Glucose" found	Calculated
(1)	None	17·7	4·8	—
	,,	35·4	9·3	—
	,,	53·0	15·2	—
	52·0	0·7	52·2	52·2
	52·0	2·8	52·7	52·8
	52·0	7·0	53·7	53·8
(2)		Creatinine		
	None	50·5	16·1	—
	50·8	0·81	51·5	51·1
	50·8	2·02	51·5	51·4
	54·4	4·04	55·4	55·7
	50·8	8·1	53·7	53·4
	50·8	20·2	57·7	57·2
(3)		Urea		
	59·6	100·0	59·1	—

	Glucose taken	Glycine	"Glucose" found	Calculated
(4)	None	20·0	None	—
	54·5	4·0	58·5	—
	54·5	8·0	62·3	—
	54·5	20·0	67·8	—
(5)		Tyrosine		
	None	50·0	None	—
	59·6	8·0	67·0	—
	59·6	20·0	71·7	—
(6)		Cystine		
	None	94·5	3·4	—
	50·7	1·9	52·7	50·8
	50·7	3·8	54·5	50·8
	50·7	7·6	54·5	51·0
(7)		Glutaminic acid		
	None	90·0	None	—
	50·6	None	50·6	—
	50·6	0·9	51·1	—
	50·6	1·8	51·8	—
	50·6	3·6	53·6	—
	50·6	9·0	54·1	—
(8)		Cystine, glutaminic acid		
	52·0	3·8 7·2	58·3	52·1

B. *Hagedorn and Jensen method. All figures given are mg. per 100 cc.*

	Glucose taken	Uric acid	"Glucose" found	Calculated
(1)	None	100	53	—
	"	500	264	—
	112	50	143	138
(2)		Creatinine		
	None	100	67	—
	"	500	282	—
	167	100	226	234
(3)		Glycine		
	167	None	167	—
	None	100	None	—
	167	100	167	—
(4)		Cystine		
	109	None	110	—
	None	100	44	—
	109	10	119	113
	109	100	132	153
(5)		Glutaminic acid		
	109	None	108	—
	109	50	108	—
	109	100	109	—
	164	100	164	—
	167	100	167	—

With regard to the Wood-Ost method, the following points may be noted. In the case of uric acid, a considerable amount of copper urate was precipitated which caused some delay in filtering, but the liberated uric acid reacts only very slightly with potassium permanganate in the cold. The solutions remained blue during the reduction. Creatinine, as was pointed out by Taylor [1924], has a most remarkable power of delaying the precipitation of the red

cuprous oxide; whether due to combination with it or with the glucose appears to be uncertain. The solutions containing the larger amounts of creatinine and glucose acquired the opaque yellowish-green appearance which is seen when urine containing glucose is heated with Fehling's solution. This colour did not occur in the absence of glucose. The amount of cupric salt reduced by mixtures of glucose and either of the above substances is approximately that calculated from their separate reducing powers. Urea had a slight depressing effect, presumably due to the ammonia liberated by partial hydrolysis.

In the case of the mixtures of glucose with amino-acids, the amount of copper salt reduced was decidedly greater than that reduced by the glucose alone, even when the amino-acid alone had no reducing power. If, as in the case of cystine, a small amount of copper was reduced by the amino-acid alone, the amount reduced by the mixture of it with glucose was still greater than that calculated from the sum of their two separate reducing powers. In the presence of cystine the rate of reduction appeared to be greatly increased and the solution became somewhat greenish in colour but remained perfectly transparent. In the case of the other amino-acids, the appearance of the solution was similar to that when glucose alone was used.

With the Hagedorn and Jensen method no coupled oxidation of amino-acids could be detected. The reducing power of mixtures of glucose with either cystine, uric acid or creatinine was equal to that calculated on the assumption that each substance reduced the ferricyanide independently of the other.

In order to show, if possible, the actual decomposition of a stable amino-acid in the presence of glucose by an alkaline copper solution, 1 g. of tyrosine was heated with 5 g. of glucose and 300 cc. of Benedict's qualitative copper solution. After cooling, the liquid was extracted with ether. The ethereal extract was freed from ether by distillation and the solid remaining after removal of the ether was dissolved in a few cc. of water and carefully filtered. The filtrate gave strong Millon's and xanthoproteic reactions but was free from amino-nitrogen. This indicated that a portion of the tyrosine had been decomposed.

In order to observe the measure of agreement between the Wood-Ost and the Hagedorn and Jensen methods on some blood filtrates, the following technique was employed. The proteins of blood were precipitated with tungstic acid; the precipitate was filtered off and well washed with cold water. The united filtrate and washings were adjusted to p_H 6·5 and concentrated under diminished pressure to a small bulk. Alcohol was added to make the concentration 75 % and the precipitate of sodium sulphate, etc., filtered off and washed with 75 % alcohol. The alcohol was removed under diminished pressure and the residue dissolved in a small volume of water and filtered. In this filtrate the reducing power was estimated by the two methods. The results appear below. The examples of rabbit's blood were part of a joint experiment with Dr H. D. Kay and Mr J. T. Irving. The writer desires to acknowledge their courtesy in this matter.

Source of protein-free filtrate	"Glucose" mg. per 100 cc. Wood-Ost	H. and J.
1. Ox blood	280	250
2. Ox corpuscles	3	19
3. Sheep corpuscles	5	30
4. Rabbit's blood	900	1090
5. Rabbit's blood (convulsed)	320	600

In Nos. 4 and 5 the samples were suitably diluted before estimation.

The high values obtained are due to considerable concentration. Dr Kay isolated specimens of phenylglucosazone from No. 4 and No. 5.

The considerable divergences in the case of the blood of rabbits convulsed with insulin indicate that in this case at least unknown reducing substances are present in significant concentrations.

Discussion of Results.

The facts recorded in this paper have two main bearings on research on carbohydrates.

Firstly, they indicate the need of testing any proposed method of analysis on mixtures of pure substances as similar as possible in composition to the mixtures it is intended to analyse in the course of the research, as well as on as many individual substances as possible.

Secondly, they emphasise the fundamental lack of specificity inherent in the methods at present available for the estimation of carbohydrates. Statements, for instance, that a given method "does not estimate substances in the blood filtrate other than reducing sugars" are probably in need of experimental confirmation. Similar considerations apply to Harned's [1925] search for the "absolute value" of the concentration of glucose in blood.

The apparent absence of amino-nitrogen from his blood-filtrates may have been due to the presence therein of sodium nitrate, arising from the decomposition of the mercuric nitrate, which would interfere with the Kjeldahl method of estimation.

The recently published micro-method of Benedict [1925] is interesting in its apparently greater specificity and the use of sodium bisulphite to increase the amount of cuprous oxide reduced by a given amount of glucose. Since, however, glucose is now shown to be capable of inducing coupled oxidations, an explanation of the beneficent effect of the former substance on these lines would seem to be at least as probable as one based on any power of combining with portions of the glucose molecule under the conditions obtaining during the reduction of the copper sulphate, and hence protecting them from the action of the alkali.

All conclusions concerning the nature of the reducing carbohydrate in blood or other biological fluids based on the ratio of its polarimetric estimation to that based on copper reduction methods may be viewed with reserve.

The presence of glutathione [Holden, 1925] in blood-filtrates lowers the rotation and increases the reducing power towards many copper reagents. The problem of its estimation in these filtrates is not easy of solution. Tunnicliffe's [1925] method is not suitable owing to the slowness of filtration from the precipitated proteins. By reduction with zinc dust and acid followed by filtration and the use of Tunnicliffe's method on a blood filtrate the writer estimated it to be present to at least 0·02 % in the original blood. This would be quite sufficient to alter appreciably the reducing power of the glucose present, in addition to lowering the rotation. The estimation of glucose based on the quantitative formation of glucosazone and its subsequent titration with titanous chloride as proposed by Knecht and Hibbert [1924] is, from the point of view of specificity, most attractive, but after a prolonged trial the writer was unable to place sufficient reliance on it for purposes of research. In spite of the utmost precautions in connection with the purity of all the substances used, the results obtained were frequently far too high, owing apparently to the oxidation of a portion of the phenylhydrazine during the formation of the osazone, which oxidised portion oxidised some of the titanous chloride. The method was originally worked with a specially purified foreign brand of phenylhydrazine which is not available in England, but it is doubtful if that is the whole explanation of the difficulty.

Summary.

If glucose is estimated by a copper reduction method in a fluid containing amino-acids the value obtained may be too high, even though the amino-acids present do not alone reduce the copper solution. The error may amount to 10–15 % and appears to be due to an oxidation of the amino-acids coupled to that of the glucose.

The Hagedorn and Jensen method is unaffected by the presence of most amino-acids in moderate concentration.

If the Wood-Ost and the Hagedorn and Jensen methods be applied to the concentrated filtrate from the blood of a rabbit in hypoglycaemic convulsions following the injection of insulin, there is a great divergence between the amounts of glucose estimated to be present, due to unknown reducing substances. These methods agree more closely on normal blood-filtrates.

The writer desires to acknowledge his indebtedness to the Royal Society for a grant, and to Sir F. G. Hopkins for his continual interest and encouragement.

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XXXVI. THE ACTION OF LIGHT ON BLOOD[1].

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(*Received January 30th, 1926.*)

Introduction.

This investigation was begun with the object of determining the effect of light, if any, on the respiratory function of blood. Briefly, its effect was to interfere with the respiratory function, since, although it caused absorption or liberation of oxygen by various components of the blood, these were irreversible in character.

In order that the phenomena to be described may fall within the compass of a short paper, the presentation of hypotheses is left over to allow of a fuller treatment of the dynamics of the responses to light to which this paper is confined. Although the photo-chemical reactions appearing here are either imperfectly or not at all understood, many interesting comparisons with oxidation and reduction phenomena [Hopkins, 1925] present themselves to the biochemist and it is hoped that in his hands the easily controlled method about to be detailed will prove to be yet another useful means for the examination of intermediary processes in oxidation.

The influence of radiant energy of short wave-length upon the dissociation of CO_2 from solutions of haemoglobin and suspensions of red blood cells has been examined by Straub and Gollintzer-Meier [1923], who consider Einstein's formula to be quantitatively applicable to the change. The present paper deals only with oxygen.

Experimental methods.

The chief components of the apparatus used are diagrammatically represented in Fig. 1, the essential measuring instrument, the blood-gas apparatus of J. S. Haldane [1896, 1920], appears on the right-hand side; this was found to give very constant results when suitable precautions were adopted. The placing of a thick glass screen in front of the burette and levelling tubes as recommended by Douglas and the viewing of the fluid levels through magnifying lenses contributed greatly to constancy and accuracy in reading the burette. The latter consisted of a 1 cc. pipette graduated in large divisions of 0·01 cc. so that it could be read easily to 0·001 $\pm$ 0·0005 cc.

[1] Communicated to the Biochemical Society meeting on Dec. 7th, 1925, at South Kensington.

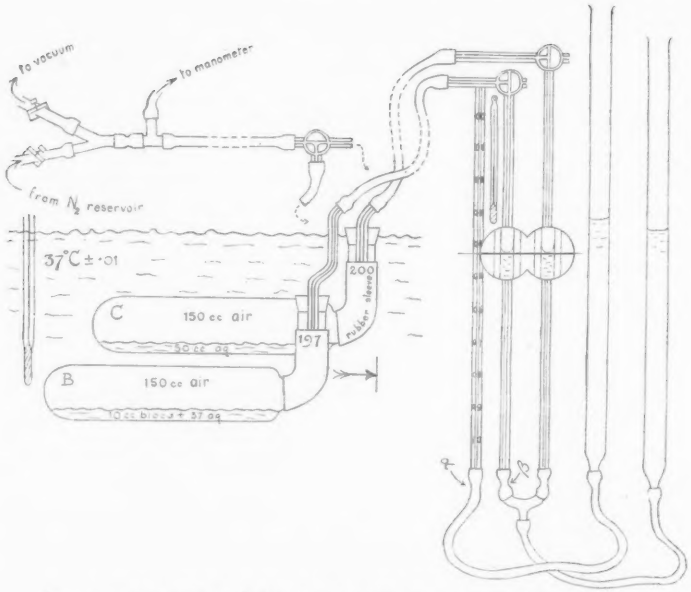

Fig. 1. The quartz blood-tube *B* and control tube *C* coupled to a Haldane constant-pressure gas volumeter.

In the earlier experiments the oxygen capacity of 1 or 2 cc. of blood was determined by the ferricyanide method, using flasks of a shape described in a previous paper [1920]. Some of these were made of quartz and others of glass covered with an electrolytic coating of copper so that a comparison of the capacities in the light and in the dark could be made. Slight differences were recorded which became more obvious with the use of a thinner burette with a maximum range of only 0·25 cc. This, however, was soon abandoned on account of its inconstancy due to varying capillarity, and a return to the 1 cc. burette was rendered practicable by working with larger quantities of fluid (10 cc.). In order to pick up as much radiant energy as possible the blood and control flasks were then made tubular in shape and of about the same length as the mercury-vapour lamp. The final form of tubes evolved is shown in the diagram (Fig. 1), in which the lower tube is seen slightly displaced to the left for clearness; both were made of quartz with a total capacity of about 200 cc. each.

The tubes were wholly immersed in an electrically heated water-bath and the temperature was maintained electrically and by hand-control at 37·0°; during the experiments the temperature was not allowed to vary more than 0·01°. A quartz window 12·5 cm. square was fitted into one side of the thermostat and a similar one of glass into the opposite side; distilled water was used in the bath and was frequently renewed since the transparency to

light of short wave-length is appreciably lowered by soluble impurities and floating particles cause much scattering of light [Pidduck, 1909]. The water was kept circulating by a suitably placed propeller and the tubes were actively rocked so as to spread the blood out in a film for maximum light absorption. All readings were made while the blood was being agitated; this is extremely important if constant penetrability and absorption of radiant energy is to be maintained.

The air spaces above the fluids in tubes *B* and *C* (Fig. 1) were always equalised; this could be checked in a preliminary experiment by arranging that large variations in temperature of the bath should not affect the burette reading.

The source of radiant energy found to be most suited to this work was an air-cooled quartz mercury vapour lamp of the vacuum type ($\frac{1}{2}$ kw.). This was placed close to the quartz window and its height adjusted by trial so that the blood and control tubes *B* and *C* (Fig. 1) were equally irradiated; this was easily determined by placing distilled water in *B* and *C* and adjusting the lamp until no change occurred in the burette reading when the light was switched on. For an approximate estimation of the efficiency of the various regions of the spectrum in eliciting the phenomena to be described glass filters in common use were inserted in turn against the quartz window inside the bath. These filters consisted of the usual photographic ruby and canary, also signal green and blue glass and lastly cobalted quartz; consequently, their use must be regarded only as a first approximation in spectral localisation.

Two goats were kept especially for the purpose of providing uniform samples of fresh blood. The usual procedure was to draw the blood from the jugular vein into a paraffined syringe and either transfer it directly to the blood-tube or centrifuge it under paraffin, using a mixture of 1 part of sodium fluoride and 3 parts of potassium oxalate as an anti-glycolytic and anti-coagulating agent.

The blood-tube *B* (Fig. 1) could be attached to a manometer and either to a vacuum pump or to a nitrogen reservoir containing commercial nitrogen kept over alkaline pyrogallol solution. In this way the gases could be thoroughly washed out by repeated exhaustion and filling of the blood-tube with nitrogen, and it was possible to admit air to give any desired oxygen tension up to 150 mm. Hg. The tubes were vigorously agitated in the dark until equilibrium was established between the blood and the gas mixture and a perfectly constant reading of the burette was obtained. Some minutes' exposure to the radiations of the mercury vapour lamp was then made and time given afterwards for return to a dark equilibrium. Samples of the gas in the blood-tube were withdrawn for analysis at the end of the experiment and the oxygen content determined in a Haldane air analysis apparatus: the CO_2 was usually inappreciable and the O_2 in close agreement with the initial tension of O_2 admitted into the apparatus in those experiments in which no transfer of oxygen occurred.

Control experiments. After the volumes of fluid in the blood and control tubes had been adjusted tests were carried out on the effects of variation in temperature of the bath, and it was found that the maximum error due to the variation permitted in the experiment was ± 0·05 mm.³, which is considerably less than the personal error in reading the burette. The height of the mercury lamp was then permanently fixed so as to have an equal effect on the tubes *B* and *C* (Fig. 1) when containing the appropriate amounts of distilled water only.

The effect of inclusion in tube *B* of salts having the same concentration as in the blood was examined. The lowest curve (Fig. 2) shows that in an atmosphere of nitrogen, Ringer's fluid maintains a state of gaseous equilibrium both in the dark and on exposure to the radiations of the mercury arc. In air a slight but negligible absorption of oxygen takes place.

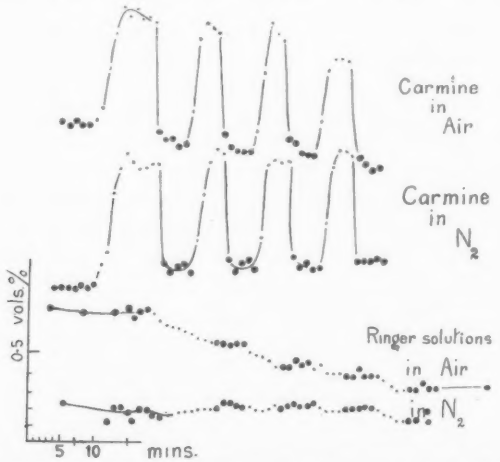

Fig. 2. Irradiation of solutions of a dye with the same colour as blood and of a solution of the saline constituents of blood.

A solution of picro-carmine matched to the colour of goat's blood and subjected to the radiations of the mercury arc shows no absorption of oxygen (Fig. 2). The two curves show the purely physical phenomenon of the local heating of the tube *B* containing this stable dye, the control tube *C* containing distilled water; the temperature of the bath, meanwhile, was maintained at 37·0°. The sharp rise in the curves (Fig. 2) after switching on the light is caused by the local heating of the tube *B* by the ultra-violet and visible radiations absorbed by the carmine and transformed into heat; the plateau represents the steady temperature maintained when the light energy absorbed is equal to the transformed heat energy radiated; switching off the light causes a drop in the curve to the original dark level. The examination of some fluorescent dyes in this manner might reveal interesting peculiarities.

Irradiation of blood.

The conditions and precise quantities of fluid used in these estimations are shown in the diagram (Fig. 1). As a rule all the radiations emitted by the mercury arc were used with the exception of the infra-red, the water in the bath completely stopping waves above $\lambda = 1{\cdot}8\mu$. Fig. 3 shows the effect on the gaseous equilibrium of blood in different tensions of oxygen of a 10-minute exposure followed by successive 5-minute exposures at intervals of 5 minutes. The sharp waves are identical with those obtained with carmine (Fig. 2) and are to be ascribed to simple physical absorption of light energy with its degradation mainly into heat. The general upward tendency of the whole curve, however, is a different phenomenon corresponding to a liberation of gas in the tube *B*. Its failure to return to the original dark level shows that we are here dealing with an irreversible reaction. The uppermost curve in Fig. 3 shows that the new equilibrium attained in the light persists for 3 hours at least.

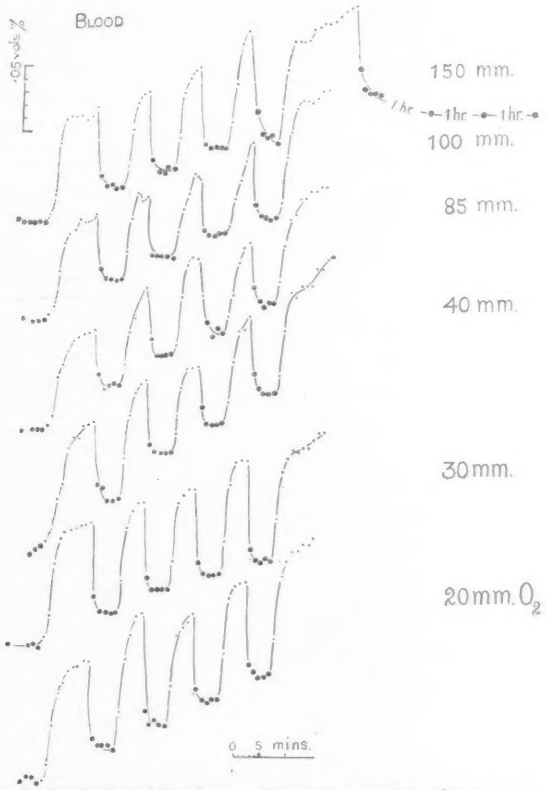

Fig. 3. Irradiation of blood exposed to various tensions of oxygen.

Fig. 4 shows the effect of irradiation at low oxygen tensions. Whereas at ensions of oxygen above 20 mm. the curves show a constant upward tendency corresponding to evolution of gas from the blood, at 10 mm. and below the curves slope downwards, indicating an uptake of gas. When tensions of oxygen intermediate between these two sets were examined the dark equilibrium was maintained practically level (Fig. 5) irrespective of exposure to light. It appeared, therefore, that either two processes were at work or two different

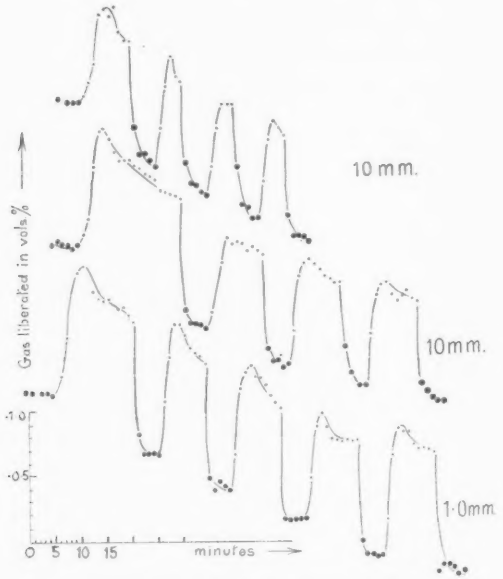

Fig. 4. Irradiation of blood at low tensions of oxygen.

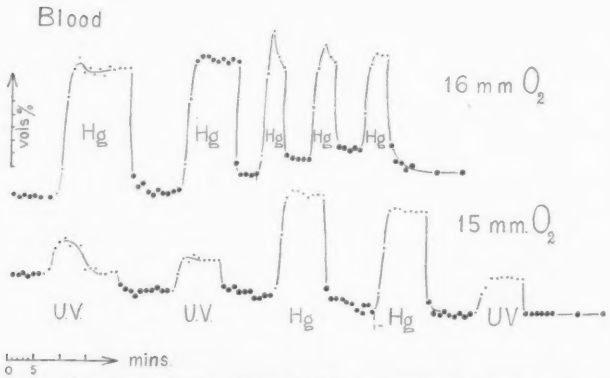

Fig. 5. Irradiation of blood at 15 mm. tension of oxygen.

constituents were involved, the one maintaining the ascendency at high oxygen tensions (Fig. 3) and the other at low oxygen tensions (Fig. 4), a balance of effects being realised at 15 mm. tension of oxygen (Fig. 5).

On separating the blood into its two components by centrifugalisation and exposing each component separately to light, it was seen (Fig. 6) that the yielding up of gas was a property of the corpuscles, whilst the taking up of gas was inherent in the plasma. That the gas taken up by the latter is oxygen may be seen from an examination of the two lower curves (Fig. 6), since if the plasma is exposed to pure nitrogen the dark equilibrium remains practically level irrespective of irradiation. It may be mentioned that the complete removal of oxygen was a matter of some technical difficulty. In these experiments the screw clips at α and β (Fig. 1) were closed so that the oxygen might be removed from the burette and its indicating fluid; this usually required from 10 to 12 washings with nitrogen. Oxygen, too, appears to be the gas evolved from the corpuscles in the top curve (Fig. 6) since no evolution takes place after the corpuscles have first been deprived of oxygen (second curve from top, Fig. 6). It appears that some reduced derivative of haemoglobin is formed under the action of light; although methaemoglobin is said to be a product of the action of light on blood this does not seem to satisfy the direction of oxygen transfer.

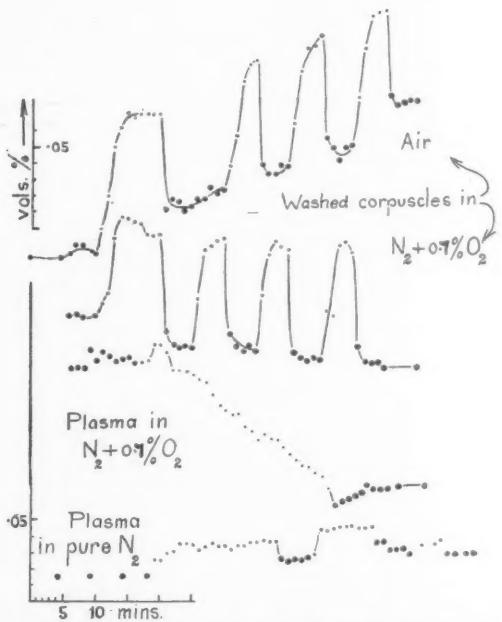

Fig. 6. Irradiation of corpuscles and plasma separately.

Oxygen uptake of plasma in ultra-violet light.

From the above experiments it appears that, provided there is a trace (0·5 %) of oxygen present in the gaseous medium in contact with the plasma, the taking up of oxygen proceeds in the light. It is immaterial whether serum or plasma be used, so that fibrinogen at least is not necessarily involved in the process. Under the conditions of these experiments serum was found to take up oxygen at a steady rate of 0·03 volume % per minute for at least half an hour.

Reference to Fig. 2 shows that the oxygen uptake of plasma in the light cannot be ascribed to the saline constituents since the absorption curve obtained with a Ringer's solution is practically level. A buffered glucose solution ($p_H = 7{\cdot}4$) took up no oxygen during fifteen minutes' irradiation. By a process of exclusion and without resort to the methods of analytical chemistry, it would appear that the constituents of plasma responsible for the oxygen uptake are either the proteins or fatty substances. That the latter, if present, should exhibit the phenomenon was anticipated especially with regard to the unsaturated forms of fat. Thus it was found that 5 cc. of a sample of cod-liver oil in 42 cc. of a buffered phosphate solution were capable of taking up 0·4 volume % per minute in the dark when the emulsion was agitated with air at 37°, whilst on exposure to the mercury lamp 9·4 volumes of oxygen % per minute were absorbed. In this case, however, the effect of the light was to accelerate 23-fold the velocity of a reaction which was already proceeding in the dark, and in this respect it differed from the effect observed in the case of plasma. Olive oil, on the other hand, in a dilution of 0·5 cc. in 46·5 cc. water showed no appreciable uptake in the dark but a very similar figure in the light, viz. 10·4 volumes of oxygen % per minute.

In order to carry the investigation of the photo-oxidation of plasma further, it became necessary to find means for amplifying the small response of 0·04 volume of oxygen % per minute. Since the effect was confined to the ultra-violet region, the possibility of extending the photo-chemical threshold into the visible spectrum was entertained. The methods of bringing this about will be described in a subsequent paper.

Summary.

Irradiation of blood with ultra-violet light causes it to give up oxygen at high oxygen tensions and to take up oxygen at low tensions. At about 15 mm. tension of oxygen, there is gaseous equilibrium, no net change occurring on passing from the dark into the light.

The taking up of oxygen by blood in ultra-violet light is a property of the plasma.

I desire to express my gratitude to Prof. A. V. Hill for valuable criticism.

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XXXVII. PHOTO-OXIDATION OF PLASMA.

A NOTE ON ITS SENSITISATION.

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(*Received February 24th, 1926.*)

Plasma can be made to take up oxygen when irradiated in the manner described in a previous paper [Harris, 1926], but the rate at which this occurs in the case of clear plasma is extremely slow. Exposure of 10 cc. of fresh goat's plasma in a dilution of 1 in 5 to the light of the quartz mercury vapour lamp ($\frac{1}{2}$ kw.) at a range of 12·5 cm. causes an absorption usually of 4 mm.^{3} of oxygen per minute, *i.e.* 0·04 volume %; at this rate it would require an exposure of 25 minutes to enable plasma to take up 1 % of its own volume of oxygen. When these volumes of oxygen are again converted into masses the magnitudes are such that the analytical chemist either loses or ignores them. However, a variation appeared in the results with different samples of freshly drawn plasma and it was suspected that the discrepancies were due to incomplete centrifugalisation. The effect of the latter can be seen in the two examples:

	Goat 1		Goat 2	
Centrifugalisation	1 hour	3 hours	1 hour	3 hours
O_2 uptake: vols. % per minute	0·065	0·030	0·060	0·029

It appears that a trace of blood-pigment may double the uptake. Irradiation of clear plasma and of a simultaneous sample which had undergone slight haemolysis gave the figures 0·04 and 0·08 respectively, another pair 0·04 and 0·07, showing that the presence of blood pigment in an amount which is only just visible may double the velocity of oxygen uptake.

As haematoporphyrin had been found to act as a powerful sensitiser in other biological experiments [Hausmann, 1910; Harris, 1925], it was decided to test whether it enhanced the oxidative action of light on plasma. Haematoporphyrin alone was first irradiated ($H\phi^n$, Fig. 1); it showed a small absorption of oxygen of the same low order as the plasma. When, however, the plasma and haematoporphyrin were together exposed to light, the rate of oxygen absorption was seen to be increased thirty- to forty-fold (Fig. 1, steep curves).

These curves (Fig. 1) reveal the characteristics common to the majority of photo-chemical reactions, viz. a period of induction lasting here nearly a minute and corresponding to the biological latent period, an intermediate

steadily maintained change or tonic state, and lastly a period of deduction. By suitably adjusting the physical conditions the induction period could be reduced to less than half a minute, which is comparatively short, so that it is unlikely that we are here dealing with an autocatalytic reaction. It must, however, be pointed out that it was impossible completely to obliterate the induction period and, as a general rule, it was found that when the duration of the induction period was 20 seconds, say, then the deduction period was 20 seconds or rather more, so that there was merely a definite lag in the response as a whole behind the irradiation.

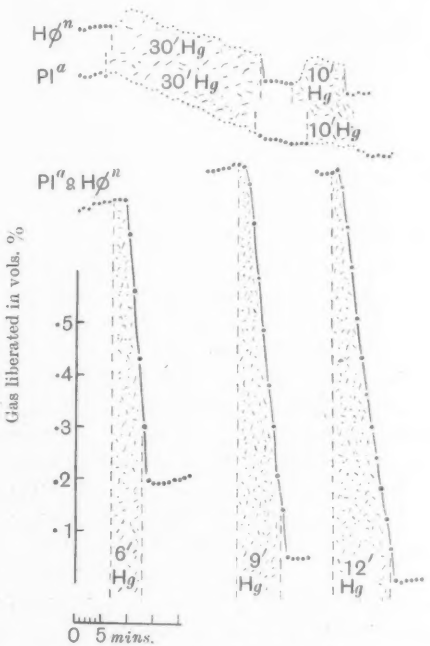

Fig. 1. Velocity of the photo-oxidation of haematoporphyrin and plasma separately and together.

The rise in the haematoporphyrin curve (Fig. 1, $H\phi^n$) shows that haematoporphyrin exhibits the absorption and re-radiation phenomenon referred to in the previous paper [Harris, 1926] and shown by any coloured substance, whereas the plasma does not do so to any appreciable extent. An examination of the plasma and haematoporphyrin curves shows that the chemical intensity of the radiations of the mercury vapour lamp increased as the lamp warmed up: it is therefore necessary in comparative experiments to run the lamp some minutes until the amperage is constant before giving an exposure.

Since the above experiments were carried out, there has appeared a preliminary notice of a method devised by Gaffron [1925] for estimating small quantities of haematoporphyrin in human serum in terms of the oxidation occurring on exposure to daylight or to the light of a metal-filament lamp; the method is said to be so sensitive that one-millionth of a gram of haematoporphyrin per cc. of serum may be detected.

In view of the light-absorbing properties and powerful optical sensitisation of chlorophyll, as well as the analogy of the position occupied by it in respect to plant life to that which haematoporphyrin holds in the animal kingdom, it was decided to test this sensitiser next. The only specimen available was a suspension in olive oil of the mixed chlorophylls from nettles. Olive oil, when agitated in water, was found to take up 10 volumes of O_2 % per minute's irradiation, whereas the olive oil and chlorophyll took up 170. When added to plasma, they caused a fifty-seven-fold acceleration in the oxygen uptake, but on account of the presence of the olive oil it was impossible to apportion the respective effects to plasma (40 ?) and oil (17 ?). The outstanding feature of the effect of chlorophyll was the extent to which the photo-chemical threshold had been shifted into the red end of the spectrum, which brings this into line with its effect on the sensitisation of silver salts in panchromatic photographic plates as well as with the optimum action to red light in photosynthesis.

Although a very pure sample of carotene was available, difficulties were encountered in measuring its photo-sensitisation on account of its great insolubility. Experiments with carotene dissolved in chloroform and also with carotene suspended in olive oil gave a small ($\times$ 2) increased rate of photo-oxidation which was probably not more than could be attributed to the oxidation of carotene itself.

A consideration of the curves of Fig. 1 shows that we are dealing with a complex set of reactions. Reference to the figure makes it clear that the dark state is one of equilibrium so that oxidation of plasma in the light does not belong to the type of photo-reaction in which the light acts as a catalyst accelerating a reaction already taking place in the dark, as is the case with cod-liver oil when agitated with air. The photo-oxidation of plasma, on the contrary, appears to involve a true endo-actinic stage, the light energy actually bringing about a change in the chemical potential rendering possible a reaction which would not otherwise occur (in the dark); the oxidation of plasma in the light becomes then a photo-chemical reaction of the simplest type. The theory of high energy chemistry recently advanced by Baly [1925] may be interesting in this connection. On the inclusion of haematoporphyrin the reacting system becomes very complicated; the net effect of this substance is to accelerate the velocity of the photo-oxidation of plasma. Whether the effect of the haematoporphyrin is simply a catalytic one might be determined by carrying the photo-oxidation of plasma to completion, using different concentrations of haematoporphyrin on equal quantities of substrate. As the present paper deals especially with the velocities of reaction, the effect of

varying concentrations of haematoporphyrin on the rate of photo-oxidation of plasma only is presented (Fig. 2). At low concentrations the curve is practically linear and the velocity of the photo-chemical reaction is directly proportional to the mass of sensitiser; at higher concentrations, a limit appears to be reached (*ca.* 0·0025 g. for 10 cc. plasma + 37 aq.) above which addition of this sensitiser has no further effect upon the velocity of reaction—a phenomenon analogous to the saturation current in photo-electricity. In all these experiments the emission of the light source was maintained constant; information relating to the energetics of the reaction might be obtained from a study of the effects of varying quantities of light upon a photo-chemical system consisting of substrate (10 cc. plasma) just saturated with sensitiser (0·0025 g. haematoporphyrin). It is interesting to note the similarity of this curve to that giving the relation between photosynthesis and light intensity [Boysen Jensen, 1918].

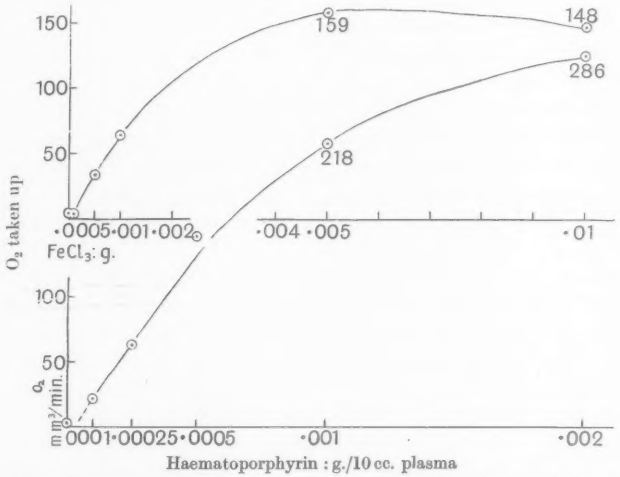

Fig. 2. Effect of concentration of sensitiser on the velocity of the photo-oxidation of plasma.

When one attempts to understand the mode of action of this photo-sensitiser, one is struck by its two outstanding optical properties. In the first place, solutions of haematoporphyrin exhibit very powerful absorption of light, as may be seen in the transmission spectrum given with the mercury arc and appearing in an earlier paper [Harris, 1925]; the absorption is nearly complete in the ultra-violet (practically all below $\lambda = 436\,\mu\mu$), and regional in the visible spectrum; it is also very marked in the infra-red, as shown by Hartridge and Hill [1914]. In the second place, on examining such solutions in ultra-violet light they display a beautiful pink fluorescence. It appears, therefore, that haematoporphyrin absorbs ultra-violet energy ($\lambda = 200 - 436\,\mu\mu$) and again radiates some of this energy in a limited spectral

band (*ca.* 0·7 μ), thus becoming self-luminous in ultra-violet. The spectral locus of this emission would be interesting in the possible applicability of the principle of resonance to the photo-oxidation of plasma alone by means of radiant energy from specific spectral bands.

The effect of a few dyes exhibiting optical properties similar to those of haematoporphyrin may be quoted:

Dye (1 % aq. solution)	Volumes of O_2 taken up by 100 vols. plasma exposed to 1 minute's radiation*					
	Ruby	Canary	Green	Blue	U-V	Hg arc/aq.
Methylene blue	0	0·15	0·06	0·11	0·25	0·67
Eosin	0	0·08	0·09	0·24	0·40	0·76
Erythrosin	0	0·69	0·66	0·53	0·75	1·77

* These figures also show that there is a good deal of overlap in these commercial glasses which are further complicated by different depths of colour.

The effects appear maximal for the regions of maximum absorption of light by the sensitiser. It is probable that fluorescence alone does not account for the difference in effect of different dyes. When the dye uranin, which exhibits a strong green fluorescence, was added to the plasma the rate of oxidation was increased only five-fold.

Turning to the chemical properties of haematoporphyrin, perhaps its most remarkable feature is the possession of an atom of iron. Upon testing a simple inorganic salt of iron in the same way as haematoporphyrin a parallel but much smaller accelerating action on the photo-oxidation of plasma was revealed. This is seen in Fig. 2, in which the two curves can be seen to belong to the type expressed by the equation $y = x^{\frac{1}{n}}$; the entire curve is given in the case of $FeCl_3$, but only the first part of the haematoporphyrin curve is shown.

The position of iron in the sensitisation of this photo-reaction appears to be unique: similar properties were not shown to be possessed by other metals producing catalytic effects in other reactions, *e.g.* salts of platinum, nickel and palladium were ineffective—very likely because they are powerful precipitants of protein. Experiments to be described later show that it is essential that the iron should be in an ionisable form and capable of passing easily from the ferric to the ferrous state—possibly by the addition of a valency electron under the action of light. In view of the less stable character of the Fe″ atom, one might expect ferrous salts to be more effective than the ferric forms; ferrous sulphate (0·1 cc. of $M/20$ solution) was found to be 33 % more powerful than equimolar amounts of ferric chloride. Ferrous ammonium citrate was then subjected to examination on account of its reputed compatibility in pharmacy and its deep colour: 0·5 g. of this salt when added to 10 cc. plasma and also to the control showed a rate of oxygen absorption expressed in volumes % per minute when exposed to the filtered radiations from the mercury vapour lamp as follows:

Ruby	Canary	Signal green	Blue	U-V	Hg arc
0	0	0	0·50	1·25	1·85

The photo-dynamic action of this salt is confined to the blue and ultra-violet which is also the spectral region of its maximum absorption.

The other scale-preparation of iron—iron and quinine citrate—behaved in a similar manner but was more sensitive, presumably on account of the peculiar optical properties possessed by the quinine, thus:

Per minute's irradiation through filters	Vols. O_2 taken up by 100 cc. plasma containing iron and quinine citrate		
	1 : 10,000	1 : 1000	1 : 100
Ruby, Canary or Signal green	0	0	0
Blue	0·09	0·71	1·59
U-V	0·30	0·75	1·70
Hg arc	0·80	0·88	2·10

This double salt may therefore be usefully employed for photo-sensitisation; it must be noted, however, that the concentration necessary to produce a given acceleration is practically ten times that of haematoporphyrin. It is interesting to speculate as to whether the extraordinary efficiency of haematoporphyrin is due to the exaggeration of the catalytic and optical properties possessed by iron and quinine respectively or to the possession of a third undefined factor. A comparison of the above compounds indicates that the more complex the ion in which the iron is joined the more effective is it as a sensitiser. Now the ferric form is very stable, whereas the bivalent form may be made to absorb oxygen from the air and link it coordinatively. When, however, the iron is joined in a complex ion it can be easily brought into each of the oxidation stages, as shown by Baudisch [1918], visible and ultra-violet radiations then possessing the power to reduce the tervalent iron of certain complex compounds into the bivalent form. Just [1907] carried out interesting investigations leading one to assume the formation of the peroxide FeO_3 as an intermediary product. Later experiments by Baudisch and Welo [1924] show that initially one molecule of oxygen is added to one molecule of ferrous salt. The oxygen molecule, thus activated, is able to link certain compounds in a coordinative manner and make them more active. Another feature of interest, if we accept the electromagnetic theory of light, is the relation of iron compounds to their state of oxidation. Hilpert [1909] has shown that white ferrous hydroxide, which is practically non-magnetic, becomes black in colour and strongly ferro-magnetic on absorption of oxygen, but on further absorption of oxygen it turns into the red and weakly magnetic ferric hydroxide. Baudisch and Welo [1924] made several observations on the affinity of the magnetic iron with the para-magnetic gas O_2 and on light absorption by complex ions, which are very interesting in connection with the experiments described in this paper.

In view of the susceptibility of the iron-plasma system to light and the extensive use made of potassium ferricyanide in estimations of the oxygen capacity of blood, it became a matter of importance to examine its effect on plasma. As it is well known that solutions of this salt rapidly deteriorate in

the light, suitable controls had to be performed, *e.g.* using 1 cc. of saturated $K_3Fe(CN)_6$ and 10 cc. plasma:

Blood-tube *B*	Control tube *C*	O_2 absorbed/min. *B*–*C*, mm.³
Plasma	H_2O	+ 6
H_2O	Ferricyanide	−22
Plasma + ferricyanide	Ferricyanide	−17

Two points are brought out here, viz. (1) ferricyanide absorbs oxygen in the light at a much faster rate than does plasma and (2) the plasma protects or removes some of the ferricyanide from oxidation. The same may be seen in the figures obtained from different controls given by the blood of another goat:

Plasma	H_2O	+ 2
Ferricyanide	H_2O	+10
Plasma + ferricyanide	H_2O	+ 5

The absence of any sensitising action by the iron in the above experiment is to be ascribed to the absence of free Fe ions from the plasma + ferricyanide system. It is, however, noteworthy that the presence of cyanide *per se* does not inhibit the photo-oxidation of plasma, as seen in the following experiment giving the number of mm.³ of oxygen absorbed by 10 cc. of plasma per minute's irradiation:

Plasma	3·0
Plasma + 0·25 g. NaCN	3·4
Plasma + NaCN + 0·1 g. haematoporphyrin	234·0

The addition of 0·25 g. sodium cyanide (CO_2-free) did not prevent the photo-oxidation of plasma nor its eighty-fold increase in velocity by the sensitiser. The system presented here must be different from the purely chemical one of Fleisch [1924], who was able to inhibit the aerobic oxidation of succinic acid by means of cyanides, or the biological system of Warburg and Meyerhof [1912], who found that the increase of oxidation by the eggs of the sea-urchin caused by the addition of iron salt is inhibited in the same way as normal respiration by cyanide and narcotics. Fleisch, however, found that the anaerobic oxidation of succinic acid which proceeds in the presence of methylene blue is unaffected by cyanides, and further Dixon and Thurlow [1924] found that the oxidation of hypoxanthine aerobically is not affected by cyanides. Corresponding to these two types of cyanide susceptibility we have the suggestion of Evans [1923] that there are two kinds of respiratory processes in plain muscle, one of which is inhibited by cyanide and the other is not. Warburg's system [1921], although containing a metallic catalyst, is susceptible to cyanide, whilst the plasma + haematoporphyrin system investigated in the present paper is insusceptible to cyanide.

Summary.

The velocity of oxidation of plasma in light may be increased fifty-fold in the presence of minute amounts of certain sensitising substances. Haematoporphyrin and chlorophyll cause a very powerful acceleration, whilst many dyes and ionic iron show a marked though somewhat smaller effect.

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XXXVIII. OBSERVATIONS ON THE VELOCITY OF THE PHOTO-OXIDATION OF PROTEINS AND AMINO-ACIDS.

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Photo-oxidation of pure proteins.

In earlier experiments [Harris, 1926, 1] it has been shown that, when blood is exposed to light, absorption of oxygen by the plasma occurs; further, that neither the saline constituents of plasma nor its glucose are responsible to any measurable extent for the uptake of oxygen. The proteins and lipins remain to be considered. As regards the proteins, since serum responds as effectively as plasma, attention may be devoted wholly to serum albumin and globulin. The only record of the effect of light on fibrinogen is that of Stalinger [1922], who found that when citrated plasma in the presence of eosin or ferrous sulphate is exposed to light the stability of the fibrinogen against a number of methods of precipitation is increased, but oxidation was not studied. In order to determine the locus of action of the photo-oxidation of plasma, the behaviour of other proteins was examined.

10 cc. of native egg-albumin suitably diluted were placed in the blood-tube [Harris, 1926, 1] in place of the usual 10 cc. of blood or plasma, and equilibrium established during agitation in the dark. On exposure to the radiations of the mercury vapour lamp after their passage through quartz and distilled water, the aqueous solution of egg-white took up oxygen at a steady rate of 17 mm^3., or 0·17 volume % per minute. Plasma under identical conditions has an oxygen uptake of only 0·04 volume % per minute, the smaller figure here being no doubt due to the lower protein content. In the experiments on egg-white it became apparent that the size of the coagulum formed in the light (the bath being maintained at 37°) also depended on the length of exposure: whether the processes of coagulation and oxygen absorption are two independent or two related effects of ultra-violet light remains to be investigated. It is interesting to note in this connection that ultra-violet light is said to increase the egg globulins at the expense of the albumins [Chalupecky, 1917].

The photo-oxidation of egg-white, like that of plasma [Harris, 1926, 2], was found to be increased by haematoporphyrin (0·01 g.):

Vols. O_2 absorbed % per min. by egg-white in presence of haematoporphyrin when exposed to the radiations of Hg arc transmitted through colour filters:

Ruby	Canary yellow	Signal green	Blue	U-V
0·01	0·17	0·24	1·04	1·28

The spectral effect is identical with the regions of maximum absorption by the sensitiser subject to the reservations regarding these colour filters mentioned in the preceding paper [Harris, 1926, 2].

As a further step in the localisation of the oxygen absorption, pure proteins were examined. A crystalline preparation of ovalbumin subjected to examination responded as follows:

Vols. O_2 absorbed % per minute:

10 cc. 5 % ovalbumin	0·06
Ovalbumin + haematoporphyrin 0·01 g.	2·30

figures which are fairly close to those of goat's blood plasma for a like concentration of albumins + globulins.

A fat-free preparation of caseinogen (0·5 g.) yielded the following results on exposure to the radiations of mercury-vapour:

Vols. O_2 % per minute:

Caseinogen	0·12
Caseinogen + haematoporphyrin	1·67

The examination of a vegetable protein was undertaken with a view to determining whether proteins from plant sources behave like animal proteins and whether they respond alike to sensitisation by chlorophyll and haematoporphyrin. Crystallised edestin (0·5 g.) in aqueous solution was found to undergo photo-oxidation at a rate of 0·10 volume % per minute, which was increased to 1·54 on the addition of 0·01 g. haematoporphyrin. The effect of adding 0·1 cc. of the olive-oil emulsion of chlorophyll [Harris, 1926, 2] and exposing to various spectral regions is seen in the following figures, which give the rate of oxygen uptake in volumes % per minute:

Ruby	Canary	Green	Blue	U-V
0·06	0·18	0·24	0·66	0·85

It is seen that the effect of chlorophyll extends more powerfully into the red region of the spectrum. These figures must not be interpreted as representing true actinic values for reasons already given [Harris, 1926, 2]. Accurate actinometric measurements, while using specific chlorophylls, would be interesting in their possible relation to the rôle of this photo-sensitiser in photosynthesis.

An outstanding exception to the photo-oxidation of proteins is that of gelatin. A highly purified sample in 2 % aqueous solution showed no absorption of oxygen in the light of the mercury arc; the figures were:

Vols. O_2 % per minute:

Gelatin 1 g.	0·00
Haematoporphyrin	0·03
Gelatin + haematoporphyrin	0·03

The transparency of gelatin solutions might be advanced from an optical standpoint as a partial explanation; even so, the addition of a highly absorptive sensitiser would be expected to activate it. It is more likely that the failure

of this protein to fall into line with those examined above is due to its exceptional constitution with respect to the particular amino-acids contained in it.

Photo-oxidation of amino-acids.

The amino-acids, when dissolved in distilled water and agitated with air, resolved themselves into two well-defined groups as regards the action of light. The following, in amounts of 0·2 g. each, effected no appreciable change in the volume of air in contact with their solutions: glycine, alanine, valine, leucine, asparagine, aspartic acid, histidine (HCl).

Tryptophan and tyrosine, on the other hand, were extremely sensitive to light under similar conditions:

O_2 taken up per minute, mm.^{3}:

Tryptophan 0·2 g.	24
„ + haematoporphyrin 0·005 g.	96
Tyrosine: solution saturated at 37°	9
„ : hot sat. soln. cooled to 37°	20
„ + haematoporphyrin 0·005 g.	26

In view of their own photo-sensitivity, the acceleration caused by haematoporphyrin was necessarily much smaller than was the case with plasma. Since tryptophan and tyrosine constitute about 2·5 % of the amino-acids in serum globulin and albumin (Plimmer) an arithmetical computation will show that these two amino-acids are responsible for the bulk of the oxygen uptake by plasma. The presence of these two amino-acids in blood plasma and their absence from the insensitive gelatin invite very tempting deductions regarding the locus of the action of light on blood plasma, pending chemical analysis of the oxidation products formed under the action of light in all the above cases. It is interesting to note, however, on the physical side that tyrosine and tryptophan are known to be strong absorbers of ultra-violet radiation [Schmidt and Norman, 1920]. In the present experiments it was observed that what at first appeared to be a reversed phenomenon with the first group of amino-acids was due to the marked reflecting power of their solutions towards the incident radiations. This conformity of amino-acids to Draper's law of photo-chemical activity lends some support to the theory correlating light absorption with chemical constitution. It is interesting to recall the investigations of Witt [1876], who attributed colour to the existence of certain groups or radicles which he termed *chromophores*, a substance containing a chromophore being a *chromogen*; in the widest sense of the term a chromogen is a substance possessing a banded absorption in the ultra-violet or visible spectrum. A comparison of the behaviour of the amino-acids studied above seems to point to the benzene radical as the chromogen; benzene itself is photochemically very important, and in solution or as vapour exhibits seven absorption bands in the ultra-violet [Baly and Collie, 1905]. A sample of *l*-phenylalanine, isolated from silk, in a saturated solution in water, in which it is only sparingly soluble, showed a rate of absorption under the usual conditions of

these experiments of 7·5 mm.^{3} per minute in the light. An older sample, of American origin, which was a good deal more soluble, took up oxygen at a rate of 2·4 mm.^{3} per minute; this may have undergone oxidation during storage. Phenylalanine, however, was found in aqueous solution to be insensitive to light. The dihydroxy-derivative, like tyrosine, is very susceptible: thus 0·1 g. dihydroxyphenylalanine, on exposure to the radiations of mercury vapour, absorbed oxygen at a rate of 7·5 mm.^{3} per minute—this was not found to be accelerated by ferric chloride. It is a matter of interest that this substance is intimately related to melanin pigment. Groups other than phenyl therefore play an important part. Harden and Norris [1915] made the interesting observation that the reduction of methylene blue in the presence of lactic acid was greatly accelerated by light: they offered an explanation in terms of the methylene blue, but a consideration of the present experiments explains it as a photo-oxidation of lactic acid in the presence of an optically sensitising dye.

In view of the interesting findings of Hopkins [1925], cystine and cysteine were examined in some detail. They were, however, found to fall respectively into the afore-mentioned groups. Cystine, when suspended in distilled water or in buffered solutions of phosphates ($p_H = 7·4$), was found to be quite stable in the light and remained unaffected even with the addition of haematoporphyrin; it is probable that its insolubility and light-reflecting power contribute largely to this inactivity. When, on the other hand, its reduced derivative was placed in distilled water at 37°, agitation in the dark caused an uptake of 0·1 cc. of oxygen per minute per g. cysteine. This rate was already so high in the dark that irradiation with the mercury lamp increased the velocity of the uptake by only 50 % and, further, the addition of haematoporphyrin caused only a comparatively small increase. There must obviously be other limiting factors here affecting the rate of oxidation. It must be mentioned that Dixon and Tunnicliffe [1923] found that direct sunlight had a strong accelerating influence on the reduction of methylene blue by glutathione. Examination of a substance related to cysteine which is stable in the dark showed definite photo-chemical sensitivity; thus, 0·1 g. sodium taurocholate absorbed oxygen at the same rate as 10 cc. of plasma on exposure to the radiations of the mercury vapour lamp.

Summary.

A large number of proteins take up oxygen when exposed to the radiations of the mercury arc; gelatin is an exception.

Most amino-acids are insensitive to these radiations, but tryptophan and tyrosine absorb oxygen at a rapid rate in the light of the mercury vapour lamp. Cystine is quite stable (insoluble), but cysteine takes up oxygen so rapidly in the dark that the acceleration produced by light is small and negligible when compared with that of tryptophan and of tyrosine.

I wish to express my thanks to Dr Chibnall and Prof. Drummond for providing me with pure proteins and amino-acids for these preliminary tests.

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XXXIX. CHEMISTRY OF THYROXINE.

I. ISOLATION OF THYROXINE FROM THE THYROID GLAND.

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In 1919 Kendall [1919, 1] announced, as the result of a long and painstaking investigation, the successful isolation from the thyroid gland of a crystalline substance containing 65 % of iodine, which he named "thyroxin," and which has been proved by numerous clinical investigations [Boothby and Sandiford, 1924] to be the substance, or at least one of the most important substances, through which the thyroid gland exerts its control of the metabolic rate. Subsequently Kendall [1919, 2] published a second paper in which the empirical formula of "thyroxin" was stated to be $C_{11}H_{10}O_3NI_3$; a number of salts and other simple derivatives of "thyroxin" were described, and finally a structural formula was proposed indicating that the substance was a derivative of α-oxindolepropionic acid in which the benzene ring was reduced and had three iodine atoms attached to the 3-, 4- and 5-carbon atoms respectively. Contained in this formula are several inherent chemical improbabilities, if not impossibilities, but it is perhaps not necessary to enter into this question further here, since a careful study of Kendall's paper reveals the very slender nature of the evidence from which the formula is deduced. Indeed, it is justifiable to say that, from a chemical point of view, there is no evidence. Two points may be mentioned in this connection. In the first place, the presence of an indole nucleus is deduced from the fact that the substance gives the pine-splinter reaction for pyrrole under conditions which practically amount to an alkaline fusion; now it is a well-known fact that many substances other than pyrrole or indole derivatives will give this reaction provided that they contain an open chain grouping of atoms which, under the influence of dehydration or oxidation, can undergo internal condensation with pyrrole ring formation; this piece of evidence, therefore, unsupported by any other in the same direction, is of no value. In the second place, the presence of a reduced benzene ring in the molecule is deduced from the percentage of hydrogen as determined by combustion; the special difficulties of accurate determination of hydrogen by combustion in a substance containing 65 % of iodine are sufficiently

obvious, but, even assuming these difficulties to be overcome, a difference of two hydrogen atoms, in a substance of the formula proposed by Kendall, makes a difference to the analytical result which is scarcely outside the ordinary experimental error.

It is fully apparent, therefore, that without considerable further chemical evidence it is impossible to accept the formula proposed by Kendall, and that the constitution of thyroxine must be regarded as not proven.

Considerations of the nature outlined above led the present writer [Harington, 1925] to make a synthetic "shot in the dark" at the constitution of thyroxine by the preparation of a substance agreeing closely in empirical formula with that deduced from Kendall's analyses, and, further, a substance which might be expected to give chemical reactions of the type which was described as characteristic of thyroxine. The compound synthesised was 3:4:5-triiodophenylpyrrolidonecarboxylic acid, and it showed no physiological action resembling that of thyroxine. The failure of this time-consuming attempt indicated the futility of such a method of attack and forced upon the writer the conclusion that further progress was not likely to be made with the elucidation of the constitution of thyroxine until sufficient supplies of the substance were available to permit of its systematic chemical degradation. The first part of the problem resolved itself, therefore, into the question of the isolation of thyroxine from the thyroid gland.

The method employed by Kendall [1919, 1] for the isolation is laborious and time-consuming in the extreme; he himself states that he worked up three tons of fresh thyroid gland with a total yield of 33 g. of thyroxine; such a method is obviously impracticable from an ordinary laboratory point of view. Thyroxine is prepared according to Kendall's method by Messrs Squibb, of New York, but, as might be expected from the technical difficulties of the preparation, the price is prohibitive even for the small amounts that are used for therapeutic purposes. It was essential, therefore, that a simpler and cheaper method of extraction should be devised.

It appeared probable, from a study of Kendall's work, that a considerable proportion of the total loss of active material occurred during the preliminary process of hydrolysis of the gland for 24 hours in 5 % sodium hydroxide, so that the first attempts were directed towards finding a substitute for this. In particular, many attempts were made to employ tryptic digestion of the gland, in the hope that the gentle but deep-seated hydrolysis so effected might bring about a more favourable separation. The first experiment in this direction did indeed appear promising, in so far that as much as 73 % of the total iodine of the gland was obtained in a soluble condition after tryptic digestion for seven days; but the proportion of the soluble iodine which could be precipitated with acid was no larger than after hydrolysis with sodium hydroxide; larger amounts of iodine could be precipitated with metallic precipitants (*e.g.* mercuric sulphate in almost neutral solution), but the recovery of the iodine-containing compounds from such precipitates was not

satisfactory. Attempts were then made to effect a further separation by prolonged tryptic digestion of the acid-insoluble products of the preliminary digestion and, finally, to carry the process still further by the use of erepsin. In no case, however, was it found possible to separate thyroxine from the products of digestion. It was early found that continuous extraction with butyl alcohol, under reduced pressure, of the almost neutral solution of the acid-insoluble products of the first digestion, removed therefrom a small proportion of iodine-containing material which gave the reaction with nitrous acid described by Kendall as characteristic for thyroxine, and it was hoped that the application of this method of extraction to the products of further digestion might yield a substance which could be crystallised. It was, however, found that neither the relative amount nor the purity of the substances extracted by butyl alcohol was much increased by the more prolonged digestion. Further, it was constantly found in these digestion experiments that more or less of the iodine was split off in inorganic form; although as yet no experiments have been made with regard to the action of the enzymes on pure thyroxine, it would appear probable that we have here to deal with a phenomenon similar to that encountered by Oswald [1909] in his work on 3:5-diiodotyrosine. Reflection upon the latter possible similarity led to the idea that the hydrolytic agent found by Oswald to be most suitable for the isolation of diiodotyrosine, namely barium hydroxide, might find a favourable application in this case also. The direct hydrolysis of thyroid gland with high concentrations of barium hydroxide, similar to those employed by Oswald, led apparently to much destruction of thyroxine, since, although a large proportion of the iodine was found in the filtrate after such a hydrolysis, practically none of this iodine could be precipitated with acid. Hydrolysis with dilute barium hydroxide, however, appeared at once to be more favourable since, after such treatment, the filtrate contained 60–70 % of the total iodine, of which about 40 % was precipitated, on acidification, in the form of a cream-coloured flocculent precipitate, which contrasted pleasantly with the dark, almost black precipitate obtained similarly after hydrolysis with sodium hydroxide. Both this precipitate and the insoluble material resulting from the first hydrolysis gave the nitrous acid reaction for thyroxine, whilst the reaction was absent from the filtrate after acidification. Attempts were first made to purify this precipitate further by the method described by Kendall, but iodine determinations indicated such heavy losses at the various stages that this was soon abandoned. Extraction with butyl alcohol of the ammoniacal solution of the precipitate removed about 70 % of the iodine, but even from the material so extracted it was not possible to separate thyroxine. It therefore seemed probable that further hydrolysis was required and the acid precipitate was therefore subjected to more intensive treatment with barium hydroxide; this led to a further separation of the iodine, approximately 50 % of which remained in solution, the remainder being carried down with the insoluble barium salts. The portion which remained in solution was not precipitated with acid. On

recovery from the barium salts by boiling with alkaline sodium sulphate solution and reprecipitation with acid under certain conditions, a precipitate was obtained, which, on solution in alkaline alcohol and precipitation with acetic acid, gave a partially crystalline product containing over 63 % of iodine. On further purification this yielded a substance, containing 65·3 % of iodine, which, from its physical properties and physiological action, was evidently thyroxine. Subsequently it was found that, by extracting the insoluble precipitate, obtained after the preliminary hydrolysis, with alkaline sodium sulphate, precipitating with acid, and subjecting this precipitate to the treatment above outlined, a further crop of thyroxine could be obtained. The total amount of crude crystalline thyroxine obtained by this method amounted to 1·2–1·3 g. per kg. of desiccated thyroid gland, or a little over 1 g. of completely purified thyroxine. The desiccated gland employed contained about 0·55 % of iodine; taking 0·12 % of thyroxine as an average yield, this is equivalent to 0·078 % of iodine. A little over 14 % of the total iodine of the gland was, therefore, obtained in the form of thyroxine. This sample of gland, in the fresh condition, weighed approximately 4·4 times as much as in the dry state; the yield of thyroxine calculated on the fresh gland is, therefore, 0·027 %; Kendall's yield of 33 g. from 3 tons (3000 kg.) is about 0·0011 %.

The pure substance, on analysis, yielded figures similar to those obtained by Kendall except for the nitrogen, which was found to be lower than he states, and indeed the whole analysis leads to the empirical formula $C_{15}H_{11}O_4NI_4$ instead of the formula $C_{11}H_{10}O_3NI_3$. The nitrogen analysis was confirmed on a sample of Squibb's preparation of thyroxine, and the final confirmation will be found in the succeeding paper on the iodine-free derivatives of thyroxine. In view of the self-evident identity of the substance prepared by the above method with Kendall's thyroxine, and the proof just mentioned that his proposed empirical formula is incorrect, no further reference will be made to his subsequent publications, and the work here reported is to be regarded as proceeding, from this point onwards, upon first principles.

Experimental.

Isolation of thyroxine from the thyroid gland. 500 g. of desiccated thyroid gland are boiled for 5–6 hours, under a reflux condenser, with 5000 cc. of a 10 % solution of crystalline barium hydroxide. The length of time of the preliminary hydrolysis does not appear to make much difference either to the yield of acid-precipitable iodine or to the purity of the precipitate. It is, however, of importance that the concentration of barium hydroxide should not exceed that named above. At the end of the hydrolysis the solution is allowed to stand overnight and is then filtered from the heavy dark precipitate (B) through large Buchner funnels; the clear yellow filtrate is acidified with hydrochloric acid until barely acid to Congo-red, and the light-coloured flocculent precipitate (A) allowed to settle; the insoluble material (B) is set aside to be worked up subsequently. After standing for some hours, precipitate

(A) is separated by decantation and filtration, and is washed on the filter with water, the filtrate and washings being rejected; it is then dissolved in 250 cc. of water with the aid of ammonia, treated with crystalline barium hydroxide to make a concentration of 40 % of the latter, boiled to drive off the excess of ammonia and heated for 18 hours on a steam-bath under a reflux condenser; the temperature during this period must not exceed 100°; attempts to quicken the process by boiling for shorter periods met with no success. At the end of this period the solution is filtered hot through a Buchner funnel and the precipitate (C) washed with a little hot water. The filtrate is of no further value as regards thyroxine; it apparently contains some tryptophan (positive glyoxylic and bromine reactions). The precipitate (C) is ground up and suspended in 1 % sodium hydroxide (250 cc.), the solution is boiled, and, while boiling, treated with a strong solution of sodium sulphate in slight excess; the boiling is continued for a few minutes and the solution then filtered from barium sulphate; the filtrate is brought to the boil and, while boiling, is treated with 50 % sulphuric acid until just acid to Congo red; on continuing the boiling for a minute or two the character of the precipitate changes; at first light and flocculent, it becomes heavy and granular; as soon as this change has occurred the boiling is discontinued and the solution cooled. The heavy precipitate is filtered off and dissolved in about 20 cc. of *N* sodium hydroxide, alcohol is added to about 80 % concentration, the solution is filtered from a small amount of tar, brought to the boil and treated with 10 cc. of 33 % acetic acid; thyroxine thereupon separates in a partially crystalline condition. Yield 0·4 g. (0·08 %). The material (B) is worked up as follows: the precipitates from the preliminary hydrolysis of 2000 g. of thyroid are ground up and boiled with 2000 cc. of 2 % sodium hydroxide, a slight excess of sodium sulphate being added to the boiling solution; the filtered solution is acidified with hydrochloric acid, and the precipitate is subjected to subsequent treatment precisely similar to that described for (A) above. Yield 0·9 g. (0·045 %). The total yield is, therefore, 0·125 % of the desiccated gland. The crude thyroxine so obtained may be further purified by repeated solution in alkaline alcohol and reprecipitation with acetic acid; it tends, however, obstinately to retain coloured impurities; the final purification is best effected (though with slight loss) by making use of the insolubility of the sodium salt, a phenomenon already observed by Kendall. The crude material, after one recrystallisation by the above method, is dissolved in boiling 0·5 % sodium carbonate, about 75 cc. being required for 1 g. On cooling, a heavy white micro-crystalline precipitate of sodium salt separates out. After standing overnight in the ice-chest this is separated on the centrifuge, dissolved in 80–90 % alcohol with the aid of a little sodium hydroxide and the boiling solution treated with 33 % acetic acid in excess. The thyroxine so obtained is perfectly white and crystallises in rosettes and sheaves of fine needles, as indicated by Kendall for the "keto form" of thyroxine. On heating, it darkens at 220° and melts with decomposition and evolution of iodine at 231–233°;

this melting point is lower than that recorded by Kendall, but a sample of Squibb's "thyroxin crystals" behaved similarly, darkening at 220° and melting with decomposition and evolution of iodine at 229–231°; a mixture of the two preparations melted at 231–232°. The pure thyroxine is insoluble in water and in all the ordinary organic solvents. It is soluble in the cold in solutions of the alkali hydroxides, provided that the concentration of alkali be not too high; it is precipitated in an amorphous condition from its solution in alkali by the addition of acid, in excess of which it is not soluble. It dissolves in sodium carbonate solution only on boiling. It is soluble in 90 % alcohol in the presence of either alkali hydroxide or of mineral acid, but not in presence of acetic acid.

A solution of 0·75 g. thyroxine in 5 cc. *N* sodium hydroxide and 15 cc. of alcohol was examined in a 1 dm. tube in the polarimeter; no signs of optical activity could be observed[1]. This is in agreement with the observation of Kendall [1919, 2]. In view, however, of the process of isolation involving, as it does, prolonged alkaline hydrolysis, this cannot be taken as evidence of the absence of an asymmetric carbon atom, since, if the asymmetric carbon atom were the α-carbon of an α-amino-acid, the compound would probably have been more or less completely racemised. In view of the constitution of thyroxine, as described in the succeeding paper, racemisation during the alkaline hydrolysis is the probable explanation of the absence of optical activity in the product.

Analyses.

0·2504 g. gave 0·2125 g. CO_2; 0·0392 g. H_2O.
24·6 mg. gave 0·46 mg. N (micro-Kjeldahl).
(Squibb's "thyroxin crystals": 20·8 mg. gave 0·39 mg. N.)
0·1570 g. gave 0·1898 g. AgI (Carius).

	C	H	N	I
Found	23·14	1·74	1·87	65·3
Calculated for $C_{15}H_{11}O_4NI_4$	23·18	1·42	1·80	65·3

For final identification a physiological test was made as follows: 10 mg. of the substance were administered intravenously to a normal individual with a basal metabolic rate of − 5 %; at the end of 48 hours the basal metabolic rate had begun to rise; on the fifth day it reached its maximum of + 40 %, the pulse rate having risen from 72 to 120; the effects continued to be noticeable for a fortnight after the injection; the whole reaction was, therefore, typical of those which have been described by the American workers with thyroxine.

For assistance in making this physiological test I am indebted to Dr K. S. Hetzel.

[1] I am indebted to Mr H. J. Channon for kind assistance in making this determination.

SUMMARY.

A method is described by which thyroxine may be isolated from the thyroid gland in a yield of about 0·12 % of the dried gland.

The probable empirical formula of thyroxine is $C_{15}H_{11}O_4NI_4$.

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XL. CHEMISTRY OF THYROXINE.

II. CONSTITUTION AND SYNTHESIS OF DESIODO-THYROXINE.

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The method for the isolation of thyroxine described in the preceding paper brought the preparation of comparatively large quantities of the material within the bounds of possibility. For help in the large scale preparation of thyroxine for the purpose of the chemical work here recorded, I am greatly indebted to Messrs The British Drug Houses, Ltd., and in particular to their director, Mr F. H. Carr, under whose supervision about 100 g. of crude crystalline thyroxine have been prepared for me.

In attacking the general problem of the chemical constitution of thyroxine, it was obviously desirable in the first instance to obtain the substance free from iodine, so that the first experiments were directed towards this end. The iodine is readily removed from thyroxine by the usual reducing agents, *e.g.* sodium amalgam or aluminium-mercury couple, but in these cases the reaction products are not easy to obtain pure. A satisfactory solution was found in the use of the palladium hydroxide-calcium carbonate catalyst of Busch and Stöve [1916]; on shaking a 1 % solution of thyroxine in *N* potassium hydroxide in an atmosphere of hydrogen, using the above catalyst, the iodine was rapidly and quantitatively split off as potassium iodide and an almost theoretical yield was obtained of iodine-free thyroxine (to be called hereafter desiodo-thyroxine). This method for the removal of the iodine had the great advantage that, by performing the experiment quantitatively, it was possible to ascertain whether the hydrogen taken up was equivalent to the iodine liberated (*i.e.* 4 mols. per mol. of thyroxine), or whether it amounted to more than this quantity. Such an experiment, recorded in the experimental part below, indicated that the hydrogen taken up was indeed equivalent to the iodine split off, and it is therefore safe to assume that the removal of the iodine had taken place without the induction of secondary reductive changes within the molecule. The desiodo-thyroxine so obtained proved to have the empirical formula $C_{15}H_{15}O_4N$; it gave Millon's reaction and the ninhydrin reaction, and yielded the whole of its nitrogen with nitrous acid in Van Slyke's apparatus; it formed salts with acid and alkali; it was probably, therefore, an

α-amino-acid with at least one phenolic group. The proportions of carbon and hydrogen suggest the presence of two benzene rings.

On fusion with potassium hydroxide, the products varied with the temperature. On fusion at 250° in an open vessel, the products were *p*-hydroxybenzoic acid, a substance $C_{13}H_{12}O_2$, and a minute amount of quinol, together with ammonia and oxalic acid; on fusion at 310° in an atmosphere of hydrogen there were obtained *p*-hydroxybenzoic acid and quinol in good yield, and again ammonia and oxalic acid; the substance $C_{13}H_{12}O_2$ possessed apparently only one phenolic group (it gave a monobenzoyl derivative), the second oxygen being inert. These experiments in the first place reinforce the suggestion of the presence of two benzene rings, one at least of which has a phenolic or phenol ether group in the *para*-position to a side chain from which a two carbon fragment is split off as oxalic acid; it is unsafe to draw rigid conclusions as to the origin of the quinol, owing to the possibility of the introduction of an additional phenolic group or the production of isomeric change during the fusion.

On exhaustive methylation, desiodo-thyroxine behaved similarly to tyrosine in that the completely methylated product (betaine) lost trimethylamine with ease on boiling with alkali, and gave an unsaturated acid, $C_{16}H_{14}O_4$; methoxyl determinations on this acid indicated one methoxyl group. On oxidation with potassium permanganate (3O) this acid gave a neutral substance, $C_{14}H_{12}O_3$, together with oxalic acid. The substance $C_{14}H_{12}O_3$ gave a semicarbazone and a phenylhydrazone; since its formation in the above oxidation occurred in good yield and was accompanied by the formation of practically no acidic products, it was at first thought to be a ketone; further investigation showed, however, that the substance obtained by the action of phosphorus pentachloride on its oxime was a nitrile and not the expected anilide; the oxidation product was therefore an aldehyde, and it was then found that on further oxidation with potassium permanganate it yielded an acid, $C_{14}H_{12}O_4$, which was stable to further boiling with alkaline permanganate. Reviewing the results up to the present point then, we have in the first place by the potash fusion demonstrated the probable presence in desiodo-thyroxine of two benzene rings. The behaviour of the compound on exhaustive methylation proves almost with certainty that it is an amino-acid; moreover, the presence of one methoxyl group in the unsaturated acid $C_{16}H_{14}O_4$ proves the presence of one phenolic group only in desiodo-thyroxine; finally, the splitting off of oxalic acid by oxidation, with the formation of a residual stable acid is evidence of the presence of a three carbon side chain; on the experiments hitherto described the degradation may be represented thus:

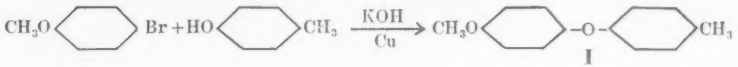
$$HO.C_{12}H_8O.CH_2.CHNH_2.COOH \rightarrow CH_3O.C_{12}H_8O.CH:CH.COOH$$
$$\rightarrow CH_3O.C_{12}H_8O.CHO \rightarrow CH_3O.C_{12}H_8O.COOH$$

The remaining question is therefore the character of the two benzene ring group —$C_{12}H_8O$—. The two benzene rings composing this group cannot be linked through a carbon atom, since such a linkage would have led, on the above scheme of degradation, to the formation of a ketone (benzophenone

derivative) on oxidation in place of the aldehyde actually obtained. They must therefore be linked either directly (diphenyl) or through the remaining oxygen atom which is as yet unaccounted for. This indifferent oxygen atom would be difficult to account for on the diphenyl hypothesis, so that the existence of an oxide linkage between the two benzene rings seemed the most probable supposition. At this point, therefore, it was decided to attempt to meet the degradation by synthesis, proceeding on the hypothesis that the group —$C_{12}H_8O$— represented diphenyl ether minus two hydrogen atoms.

On condensing *p*-bromoanisol by the method of Ullmann and Stein [1906] with the potassium salt of *p*-cresol in presence of copper bronze there was obtained 4-(4′-methoxyphenoxy) toluene (I, p. 303); this compound, on boiling with hydriodic acid, gave 4-(4′-hydroxyphenoxy) toluene (II) which proved to be identical with the substance $C_{13}H_{12}O_2$, which resulted from the mild potash fusion of desiodo-thyroxine. Further, on boiling with permanganate, the 4-(4′-methoxyphenoxy) toluene gave an acid, 4-(4′-methoxyphenoxy) benzoic acid (III), which was identical with the acid $C_{14}H_{12}O_4$ obtained, as described above, as the final oxidation product of desiodo-thyroxine after methylation.

p-Bromoanisol was then condensed similarly with potassium phenate, giving (4′-methoxyphenoxy) benzene (IV); from this, by Gattermann's hydrocyanic acid method, was prepared an aldehyde. This aldehyde was identical with the aldehyde $C_{14}H_{12}O_3$ obtained in the degradation, and was further proved to be 4-(4′-methoxyphenoxy) benzaldehyde (V) by the fact that, on oxidation, it gave an acid identical with the 4-(4′-methoxyphenoxy) benzoic acid (III) obtained as described above by oxidising 4-(4′-methoxyphenoxy) toluene. From this aldehyde it was then possible to synthesise 4-(4′-methoxyphenoxy) cinnamic acid (VI) and also desiodo-thyroxine itself. By Perkin's reaction the unsaturated acid was obtained and identified with the degradation product $C_{16}H_{14}O_4$. Desiodo-thyroxine was synthesised by two methods; (*a*) by the method of Sasaki [1921]: 4-(4′-methoxyphenoxy) benzaldehyde was condensed with glycine anhydride in presence of acetic anhydride and sodium acetate; on boiling the condensation product with hydriodic acid and red phosphorus it underwent simultaneous reduction, hydrolysis and demethylation with formation of *β*-[4-(4′-hydroxyphenoxy) phenyl]-*α*-aminopropionic acid (VII); (*b*) by the method of Wheeler and Hoffmann [1911]: 4-(4′-methoxyphenoxy) benzaldehyde was condensed with hydantoin and the condensation product boiled with hydriodic acid and red phosphorus in the same way. In both cases the synthetic amino-acid was identical with the natural desiodo-thyroxine; the proof is therefore complete that desiodo-thyroxine is *β*-[4-(4′-hydroxyphenoxy) phenyl]-*α*-aminopropionic acid, or, more simply, the *p*-hydroxyphenyl ether of tyrosine, and, further, that thyroxine is a tetraiodo-substituted derivative of this compound. The orientation of the iodine atoms in thyroxine remains to be finally determined, but experiments, which are as yet incomplete, indicate that the iodine atoms probably occupy the 3, 5, 3′, 5′ positions.



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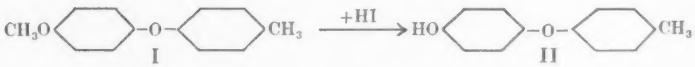

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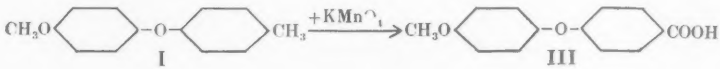

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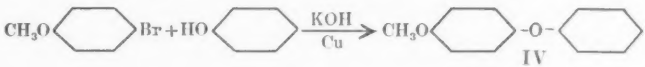

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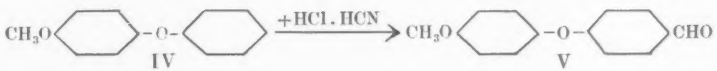

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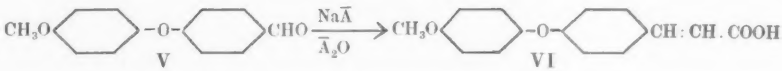

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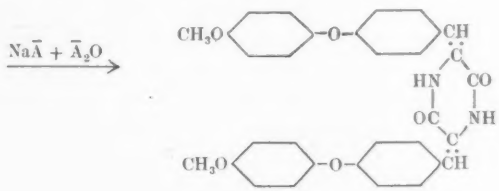

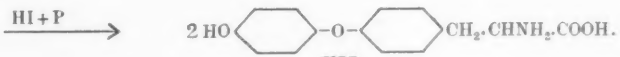

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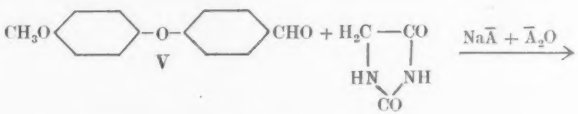

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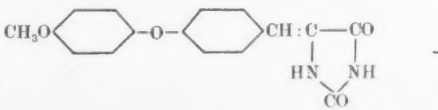

VII

Experimental.

A. *Degradation.*

Reduction of thyroxine. 6·0 g. thyroxine were dissolved in 600 cc. of *N* potassium hydroxide. To the solution were added 18 g. of a catalyst prepared by adding 1 g. palladium chloride to a hot aqueous suspension of 50 g. pure calcium carbonate, stirring well, filtering, washing until free from halogen and drying in a desiccator over potash. The solution was then vigorously shaken in an atmosphere of purified hydrogen. After a slight preliminary lag the hydrogen was rapidly absorbed, the end of the reaction being marked by the blackening of the catalyst and a sudden slackening in the uptake of hydrogen. As will be seen from the quantitative experiment recorded below, the uptake of hydrogen corresponded closely to the theoretical 4 mols. At the end of the reaction the catalyst was filtered off and the filtrate acidified with acetic acid; a small flocculent precipitate was removed by filtration and the solution evaporated on the water-bath until crystals began to separate. The solution was then allowed to stand in the cold for some time and the precipitate filtered off; the product so obtained, which was slightly pigmented, but crystalline and almost pure, amounted to 1·85 g. (88 % of the theoretical); it contained no iodine. The product could be recrystallised by dissolving in warm dilute sulphuric acid and treating the hot solution with sodium acetate until no longer acid to Congo red. Even when analytically pure it had a marked tendency to separate in spheres, which showed crystalline structure if it were crystallised rapidly or from concentrated solution. On slow crystallisation from dilute solution it formed bunches of thick needles or prisms. The melting point varied greatly with the rate of heating. On fairly rapid heating the substance melted and decomposed about 253–254°. It formed salts with acids and alkalis, the acid salts being somewhat insoluble in cold water; both acidic and basic salts were soluble in alcohol. The substance itself was almost insoluble in water, alcohol and other organic solvents; it gave a positive ninhydrin reaction, a positive Millon's reaction and a deep orange colour with Pauly's reagent; the glyoxylic reaction was negative; with nitrous acid it gave a deep orange colour, which did not, however, turn pink on adding ammonia as is the case with thyroxine. As will be seen from the analyses below the whole of the nitrogen is in the form of amino-nitrogen. From these observations and from the results obtained on analysis it appeared that the compound was an α-amino-acid with one or two phenolic groups.

Analysis. (*a*) 0·1203 g. gave 0·2893 g. CO_2; 0·0613 g. H_2O.
(*b*) 0·1182 g. gave 0·2850 g. CO_2; 0·0587 g. H_2O.
6·66 mg. gave 0·33 mg. N (Micro-Kjeldahl).
16·65 mg. gave 1·4 cc. moist N at 19° and 764 mm. (Van Slyke).

	C	H	N	NH_2-N
Found	65·6, 65·7	5·65, 5·5	5·0	4·85
Calc. for $C_{15}H_{15}O_4N$	65·9	5·5	5·1	—

The *hydrochloride* was prepared by dissolving reduced thyroxine in hot 1 : 1 hydrochloric acid from which it crystallised on cooling; it showed the same marked tendency to separate in sphaero-crystals. Recrystallised from dilute hydrochloric acid, it formed colourless thin plates, M.P. 237–239° with decomposition.

0·1274 g. gave 0·0587 g. AgCl

Found	Cl	11·4 %
Calc. for $C_{15}H_{16}O_4NCl$	Cl	11·5 %

A quantitative reduction of thyroxine was carried out as follows: 0·150 g. thyroxine was dissolved in 15 cc. *N* potassium hydroxide and treated with 0·5 g. of the palladium catalyst; the bottle containing the solution, after the air had been displaced by hydrogen, was connected with a burette containing hydrogen, the uptake of which could then be measured accurately; after shaking for 75 minutes hydrogen ceased to be absorbed; the amount taken up was 22·2 cc. at 18° and 754 mm. A blank experiment was then immediately run, the same amount of potassium hydroxide and catalyst being shaken for the same period; the uptake of hydrogen was 3·0 cc. The net uptake of hydrogen was therefore 19·2 cc. or 1·576 mg.; 150 mg. thyroxine require 1·545 mg. hydrogen for 4 mols. The uptake was therefore 2 % in excess of the theoretical. After isolation of the reduction product the filtrate and washings from the latter were treated with nitric acid and silver nitrate; the silver iodide amounted to 0·1772 g. corresponding to 0·0958 g. iodine or 97·8 % of the theoretical. It therefore seems justifiable to assume that "desiodo-thyroxine" was thyroxine in which the iodine had been replaced by hydrogen without the induction of any secondary changes.

Potash fusion of desiodo-thyroxine. (1) 1 g. desiodo-thyroxine was finely powdered and stirred into a melt of 5 g. potassium hydroxide and 0·75 cc. water in a nickel crucible which was held in a bath at 250–260°; the fusion was continued until the obvious reaction was over and the melt was becoming thick and pasty (about 45 minutes); during the fusion ammonia was evolved and a slight smell of indole was observed. The melt, after cooling, was dissolved in 100 cc. of water and the clear dark red solution saturated with carbon dioxide. A crystalline precipitate (*a*) separated and, after standing overnight in the ice-chest, was filtered off. The mother liquor was then extracted with ether, which on drying and evaporating left a crystalline residue with a strong phenolic odour (*b*). The aqueous solution was then acidified with hydrochloric acid and again extracted with ether; this ether extract yielded a dark red solid residue (*c*). The aqueous solution contained oxalic acid.

The precipitate (*a*) was sublimed *in vacuo*; the white sublimate had M.P. 56°, on recrystallisation from ligroin it formed large glistening white plates which melted at 72–73° and, on re-heating, at 56°; the substance was, in fact, apparently dimorphic, heating followed by rapid crystallisation giving the low melting point form, and slow crystallisation from a solvent the high melting form. The compound was slightly soluble in hot water, insoluble in

cold water and easily soluble in all organic solvents except light petroleum. It gave Millon's reaction.

Analysis. 3·162 mg. gave 8·945 mg. CO_2; 1·80 mg. H_2O.
Found: C, 77·42 %; H, 6·37 %[1].

Molecular weight by Rast's camphor method.
5·1 mg. in 63·5 mg. camphor. Depression of melting point 15·5°.
M.W. = 207.

The analysis and molecular weight determination suited best the formula $C_{13}H_{12}O_2$, which has C 78·0 %, H 6·0 % and M.W. 200. The compound gave, by the Schotten-Baumann method, a *benzoyl* derivative, which, on crystallisation from light petroleum, formed bundles of thick needles, M.P. 75·5–76·5°.

Molecular weight, 5·0 mg. in 47·2 mg. camphor. Depression of melting point, 13·25°.
M.W. = 320.

It was, therefore, a mono-benzoyl derivative and the compound $C_{13}H_{12}O_2$ had probably only one phenolic group.

The second phenolic fraction (*b*) was crystallised from benzene from which it formed long needles, M.P. 167–168°; when mixed with a sample of quinol the M.P. was 168–169°; it was, therefore, almost certainly quinol. The amount was so small as to preclude the possibility of further tests. The phenolic smelling substance in this fraction remained in the benzene and was probably *p*-cresol (see below).

The acidic fraction (*c*) was carefully sublimed under reduced pressure and the sublimate crystallised from water; it formed crystals with the characteristic appearance of *p*-hydroxybenzoic acid. On heating in a test-tube, phenol was given off above the melting point.

19·9 mg. air-dried substance on heating at 100° lost 2·4 mg.

Found	$H_2O = 12·06$ %
Calc. for $C_6H_4(OH)COOH, H_2O$	$H_2O = 11·54$ %

The anhydrous substance melted at 210°; when mixed with a synthetic sample of *p*-hydroxybenzoic acid of M.P. 209°, the mixture melted at 209·5°. Fraction (*c*) was, therefore, for the most part *p*-hydroxybenzoic acid.

(2) 0·5 g. desiodo-thyroxine was powdered and mixed with 5 g. powdered potassium hydroxide; the mixture was heated for 30 minutes at 310° in a copper tube from which the air had been displaced with hydrogen and through which a current of hydrogen was passed during the fusion and while the melt was cooling. After cooling, the melt was dissolved out with hydrochloric acid containing some sulphur dioxide. There resulted a practically clear and colourless solution. This was thoroughly extracted with ether; the united ethereal extracts were shaken out with dilute sodium carbonate, dried and evaporated, yielding 0·21 g. of a phenolic fraction which crystallised but was contaminated with an oily substance. It was for the most part soluble in water; the aqueous solution was shaken up with light petroleum; the latter gave, on evaporation, a small quantity of an oil which crystallised on seeding with *p*-cresol, but was

[1] The micro-combustions recorded in this paper were carried out for me by Fein-Chemie, Tübingen, Germany.

too small in amount for complete identification. The greater part of the phenolic fraction was recovered, on concentrating the aqueous solution, in the form of long needles which melted at 169° and gave an unchanged mixed melting point with quinol; on boiling with ferric chloride the smell of quinone was perceptible and crystals of quinhydrone separated on cooling.

The sodium carbonate solution was acidified with hydrochloric acid and extracted with ether yielding 0·16 g. of an acidic fraction, which on crystallisation from water formed crystals with the appearance of *p*-hydroxybenzoic acid.

33·7 mg. air-dried substance lost 4·1 mg. at 100°.

Found	H_2O	12·2 %
Calculated	H_2O	11·54 %

The anhydrous substance melted at 208·5° and, when mixed with a sample of *p*-hydroxybenzoic acid of M.P. 210°, the mixture melted at 210°. Oxalic acid was again detected in the aqueous solution of the products of the fusion. During the fusion under these conditions ammonia was evolved but no smell of indole could be detected.

Exhaustive methylation of desiodo-thyroxine. 1·6 g. desiodo-thyroxine was dissolved in 6 cc. of 2 *N* potassium hydroxide and treated alternately with six additions of 1·2 cc. dimethyl sulphate and 2 cc. of 6·5 *N* potassium hydroxide, with vigorous shaking; at the end of the reaction the solution was slightly diluted with water and treated with potassium hydroxide to make a 2*N* concentration of the latter; it was then boiled under a reflux condenser. Evolution of trimethylamine commenced at once but did not cease for some hours; as the reaction proceeded the potassium salt of the acid which was being formed, together with some potassium sulphate, separated from the solution and was filtered off from time to time. When the evolution of trimethylamine ceased, the solution was cooled and the remaining potassium salt filtered off; the whole of the latter was dissolved in hot water and the filtered solution acidified. The acid which was precipitated was best purified by means of the sodium or potassium salt, both of which were very insoluble in cold water. Finally the acid was crystallised from dilute alcohol or from 50 % acetic acid, yielding colourless glistening leaflets which melted at 175·5°. The acid was very insoluble in water but more soluble in alcohol. It decolorised alkaline potassium permanganate in the cold.

Analysis. 3·430 mg. gave 8·940 mg. CO_2; 1·64 mg. H_2O.

		C	H
Found		71·11 %	5·35 %
Calc. for $C_{16}H_{14}O_4$		71·11 %	5·30 %

2·980 mg. gave 2·46 mg. AgI (Zeisel).

Found	CH_3O	10·91 %
Calc. for one methoxyl group	CH_3O	10·50 %

The *methyl ester*, obtained by saturating the methyl alcoholic solution of the acid with dry hydrogen chloride, formed glistening leaflets from methyl alcohol (in which it was very little soluble in the cold) having M.P. 128·5°.

Analysis. 3·548 mg. gave 9·360 mg. CO_2; 1·82 mg. H_2O.

	C	H
Found	71·97 %	5·74 %
Calc. for $C_{17}H_{16}O_4$	71·83 %	5·64 %

Oxidation of unsaturated acid $C_{16}H_{14}O_4$. 0·35 g. of the acid was dissolved in 7 cc. water with 1 cc. 2*N* sodium hydroxide and the hot solution treated gradually with 8 cc. of 5 % potassium permanganate which was instantaneously reduced; sulphur dioxide was then passed in and the resulting solution was found to contain an oil, which, on cooling and rubbing, crystallised. It was extracted with ether and the ethereal solution shaken with sodium carbonate which removed a minute amount of an acid; the residue obtained on evaporation of the dried ethereal extract was crystallised from light petroleum and formed macroscopic prisms, M.P. 59·5°.

Analysis. 3·706 mg. gave 10·020 mg. CO_2; 1·79 mg. H_2O.

	C	H
Found	73·76 %	5·40 %
Calc. for $C_{14}H_{12}O_3$	73·70 %	5·30 %

It formed a *semicarbazone*, glistening colourless plates, M.P. 210–211°, and a *phenylhydrazone*, long pale yellow needles, M.P. 135–136°. The *oxime* was prepared (since the compound was first thought to be a ketone) by boiling 210 mg. with 70 mg. hydroxylamine hydrochloride and 100 mg. barium carbonate in 2 cc. alcohol for 5 hours; the solution was filtered hot and the residue boiled out with alcohol; the alcohol was evaporated, yielding an oil, which, on the addition of water, immediately crystallised. Recrystallised from light petroleum, it formed colourless leaflets, M.P. 74–75°.

2·354 mg. gave 0·124 cc. N at 25° and 726 mm.

Found	N 5·80 %
Calculated	N 5·76 %

0·3 g. of the oxime was dissolved in 10 cc. of dry ether and treated, with cooling, with 0·5 g. of phosphorus pentachloride in small portions; the resulting deep yellow solution was shaken with ice, the ether layer separated, dried and evaporated. The residue after recrystallisation from alcohol formed colourless rhombic plates, M.P. 107·5°. On hydrolysis by refluxing with strong alcoholic potassium hydroxide, this gave exclusively an acid which had M.P. 176° and was identical with the further oxidation product $C_{14}H_{12}O_4$ (see above). This experiment finally proved the compound $C_{14}H_{12}O_3$ to be an aldehyde, the product of the action of phosphorus pentachloride on the oxime being a nitrile. The aqueous solution after separation of the aldehyde contained oxalic acid; this was isolated in one experiment and amounted to about 50 % of the theoretical (assuming 1 mol. per mol. of the acid $C_{16}H_{14}O_4$).

Acid $C_{14}H_{12}O_4$. On boiling the aldehyde $C_{14}H_{12}O_3$ with potassium permanganate in slight excess of the theoretical amount and, when oxidation was complete, passing sulphur dioxide into the solution, there was obtained an acid, which on recrystallisation from dilute alcohol formed colourless plates, M.P. 177°. This acid was stable to further boiling with permanganate.

3·268 mg. gave 8·240 mg. CO_2; 1·52 mg. H_2O.
3·136 mg. gave 2·88 mg. AgI (Zeisel).

	C	H	CH_3O
Found	68·79 %	5·20 %	12·14 %
Calc. for $CH_3O.C_{12}H_8O.COOH$	68·85 %	4·92 %	12·7 %

B. *Synthesis.*

4-(4′-*Methoxyphenoxy*) *toluene.* A mixture of 18·7 g. *p*-bromoanisol, 13·5 g. *p*-cresol, 5·6 g. potassium hydroxide and 0·1 g. copper bronze in a small flask with an air condenser, was heated slowly to 235° and maintained at that temperature for 3 hours. A vigorous reaction occurs at about 160° and it is well to heat slowly until this is over; the top of the air condenser was bent so that the mixture of bromoanisol and water, which at first distilled, might be collected. This mixture was separated, when the distillation had ceased, and the bromoanisol dried and returned to the reaction flask. At the end of the reaction, a point which was marked by a considerable slackening in the rate of refluxing, the mixture was cooled and shaken up with ether and water to which had been added excess of potassium hydroxide; the ethereal layer, after separation, was once more shaken out with dilute potassium hydroxide, then separated and dried. The residual oil, after removal of the ether, was distilled under reduced pressure; the distillate crystallised on cooling. The yield was 50–60 % of the theoretical. Recrystallised from dilute alcohol the compound formed colourless plates, M.P. 47–48°.

Analysis. 0·1306 g. gave 0·3745 g. CO_2; 0·0765 g. H_2O.

	C	H
Found	78·2 %	6·5 %
Calc. for $C_{13}H_{14}O_2$	78·5 %	6·5 %

4-(4′-*Hydroxyphenoxy*) *toluene.* On boiling the above compound for 2 hours with 10 parts of hydriodic acid (sp.g. 1·7), diluting with water and cooling, a crystalline precipitate separated out; this was extracted with ether, the ether shaken with sodium hydroxide solution and the latter separated and acidified. The precipitate was filtered off, dried and crystallised from light petroleum; it formed colourless leaflets and showed the same two melting points, 56–57° and 72–73° as the compound $C_{13}H_{12}O_2$ from the potash fusion of reduced thyroxine. Further it gave a *benzoyl* derivative which formed bunches of thick needles and prisms from light petroleum, M.P. 75–77°. It was therefore identical with the compound $C_{13}H_{12}O_2$.

Analysis. 0·1072 g. gave 0·3053 g. CO_2; 0·0575 g. H_2O.

	C	H
Found	77·7 %	5·9 %
Calc. for $C_{13}H_{12}O_2$	78·0 %	6·0 %

Oxidation of 4-(4′-*methoxyphenoxy*) *toluene.* The compound was boiled for some hours under a reflux condenser with *N* potassium permanganate. The solution was treated with sulphur dioxide, cooled and extracted with ether; the ether was shaken out with sodium carbonate; the latter gave, on acidifica-

tion, a small yield of 4-(4′-methoxyphenoxy) benzoic acid, which, on recrystallisation from dilute alcohol, formed colourless plates, M.P. 176·5°; when mixed with some of the acid degradation product $C_{14}H_{12}O_4$ the mixture melted at 176·5°.

(4-*Methoxyphenoxy*) *benzene.* This compound was prepared in a precisely similar manner to the 4-(4′-methoxyphenoxy) toluene by heating a mixture of 18·7 g. *p*-bromoanisol, 11·75 g. phenol, 5·6 g. potassium hydroxide and 0·1 g. copper bronze to 235° for 4–5 hours. In this case the reaction product could be distilled under ordinary pressure, giving a colourless mobile oil, B.P. 293–296°. The yield was 70 % of the theoretical.

Analysis. 0·1262 g. gave 0·3600 g. CO_2; 0·0730 g. H_2O.

	C	H
Found	77·8 %	6·4 %
Calc. for $C_{13}H_{12}O_2$	78·0 %	6·0 %

The compound was further characterised by the fact that on boiling with hydriodic acid it gave (4-hydroxyphenoxy) benzene, felted needles from water, M.P. 84°, a compound which had been previously prepared by Biltz [1896] in a different manner.

4-(4′-*Methoxyphenoxy*) *benzaldehyde.* 21 g. (4-methoxyphenoxy) benzene were mixed with 35 cc. benzene, cooled in ice and treated with 27·2 g. fresh and finely powdered aluminium chloride and 18 cc. of anhydrous hydrocyanic acid. The solution was contained in a flask attached to an efficient reflux condenser, the upper end of which was closed by a calcium chloride tube, and also to an inlet tube with a wide mouth through which a stream of dry hydrogen chloride could be led in; the hydrogen chloride was led in for 30 minutes at 0°, 30 minutes at the ordinary temperature, and finally for 3 hours at 40–45°; the reaction mixture was decomposed with ice and hydrochloric acid and then steam-distilled for about half an hour. After cooling, the oil was separated with the aid of ether and the ethereal solution dried; the aldehyde could be distilled under reduced pressure, but there was not much to be gained thereby owing to the small difference in boiling point between the aldehyde and the unchanged starting material. It was found better to concentrate the ethereal solution and to isolate the aldehyde by means of its insoluble bisulphite compound in the usual way. After decomposing the bisulphite compound, the aldehyde was extracted with ether and the solution dried and evaporated; the residual oil crystallised rapidly; it was drained from a small amount of adherent oil on a porous tile and was then sufficiently pure for further experiments. The yield was poor and variable, the average being 25–30 % and the highest recorded just under 50 % of the theoretical. On recrystallisation from light petroleum it formed colourless macroscopic needles or long narrow prisms. The melting point was 60·5°; when mixed with a sample of the natural aldehyde $C_{14}H_{12}O_3$ which had M.P. 59·5° the mixture melted at 60°. Under the same conditions as the natural aldehyde, the synthetic product yielded a *semicarbazone*, M.P. 211°, and an *oxime*, M.P. 75°. It was therefore completely identical with the natural degradation product.

Oxidation of 4-(4′-*methoxyphenoxy*) *benzaldehyde.* 0·45 g. of the aldehyde was dissolved in 4 cc. of acetone and treated with 0·23 g. potassium permanganate which was finely powdered and added in small portions. Slight warming occurred. When the permanganate was all used up the precipitate was filtered, washed with acetone and repeatedly extracted with water; on acidifying the aqueous extract about 0·4 g. of an acid was obtained, which on recrystallisation from aqueous alcohol formed colourless plates, M.P. 176·5°; the melting point of a mixture of this acid with the degradation product $C_{14}H_{12}O_4$ was 176°.

4-(4′-*Methoxyphenoxy*) *cinnamic acid.* 0·5 g. 4-(4′-methoxyphenoxy) benzaldehyde, 0·15 g. fused sodium acetate and 0·5 cc. acetic anhydride were heated for 18 hours at 150°; the reaction product was warmed with sodium carbonate solution and filtered from some oil. The sodium salt which crystallised out on cooling was filtered off, dissolved in hot water and the acid precipitated with hydrochloric acid. On recrystallisation from dilute alcohol it formed colourless glistening leaflets, M.P. 175·5°; when mixed with the acid $C_{16}H_{14}O_4$ the melting point was again 175·5°. The sodium and potassium salts of the synthetic acid, like those of the acid $C_{16}H_{14}O_4$, are very slightly soluble in cold water. The two acids appear, therefore, to be completely identical.

(A) *Synthesis of* β-[4-(4′-*hydroxyphenoxy*) *phenyl*]-α-*aminopropionic acid.* 2·4 g. 4-(4′-methoxyphenoxy) benzaldehyde, 0·5 g. glycine anhydride and 1·3 g. freshly fused sodium acetate were ground up together, treated with 2 cc. of freshly distilled acetic anhydride and heated in a tube attached to a reflux condenser for 6 hours at 120–130°. After cooling, the solid mass was shaken out and ground up first with water and then with alcohol. Finally, it was dried, ground up, and boiled out several times with ligroin, when it was pure enough for further experiment. The compound was extremely insoluble in all solvents; it could be crystallised from a large volume of glacial acetic acid, from which it separated in small yellow rectangular plates, M.P. 286–287°. The yield was 60 % of the theoretical.

23·0 mg. gave 1·16 mg. N (Micro-Kjeldahl).

Found	N 5·04 %
Calc. for $C_{32}H_{26}O_6N_2$	N 5·24 %

1 g. of the above compound was boiled with 10 cc. of hydriodic acid (sp.g. 1·7) and 1 g. red phosphorus for 4 hours, and then, with the addition of 1·2 g. red phosphorus, for a further 5 hours; the solution was filtered hot through asbestos, diluted with water, filtered from some tar and shaken out with ether. It was then concentrated almost to dryness *in vacuo*; the crystalline residue of the hydriodide of the amino-acid was dissolved in warm water and the hot solution treated with sodium acetate. The amino-acid crystallised out and was recrystallised by dissolving in warm dilute sulphuric acid and precipitating with sodium acetate. The yield was about 50 % of the theoretical. The product showed the same crystalline form, the same solubilities and the same reactions as desiodo-thyroxine. On heating it melted with decomposition at 253–254°.

Analysis. 22·8 mg. gave 1·1 mg. N (Micro-Kjeldahl).
18·5 mg. gave 1·72 cc. moist N at 17° and 754 mm. (Van Slyke).

	Total N	Amino-N
Found	4·8 %	5·3 %
Calculated	5·1 %	5·1 %

The *hydrochloride*, prepared in the same way as that of the natural product, formed colourless plates, M.P. 238–240°; when mixed with the hydrochloride of natural desiodo-thyroxine it melted at 237–239°.

0·1139 g. gave 0·0520 g. AgCl.

Found	Cl	11·3 %
Calculated	Cl	11·5 %

(B) 4-(4′-*Methoxyphenoxy*) *benzalhydantoin*. An intimate mixture of 4 g. 4-(4′-methoxyphenoxy) benzaldehyde, 1·72 g. hydantoin, and 4 g. fused sodium acetate was treated with 8 cc. glacial acetic acid and 0·4 cc. acetic anhydride, and boiled on the oil-bath under a reflux condenser for 3 hours. While still warm the mixture was poured into a mortar, and, when cold, was ground up with water, filtered and well washed. The yellow product was then dried, powdered and boiled out several times with ligroin. After this treatment it was pure enough for further experiment. The yield was 60 %. Recrystallised from glacial acetic acid, it formed pale yellow needles, M.P. 211°.

15·0 mg. gave 1·31 mg. N (Micro-Kjeldahl).

Found	N	8·7 %
Calc. for $C_{17}H_{15}O_5N_2$	N	9·0 %

4-(4′-*Methoxyphenoxy*) *benzylhydantoin*. The sodium compound of the above condensation product was very slightly soluble in water but was soluble in dilute alcohol. The reduction was therefore carried out as follows: 4·65 g. 4-(4′-methoxyphenoxy) benzalhydantoin were dissolved in 75 cc. 2 *N* sodium hydroxide and 200 cc. 95 % alcohol and treated with 150 g. of 2·5 % sodium amalgam which was broken into small pieces and added in five portions in the course of half an hour, the solution being well shaken; after standing for an hour, 100 cc. of water were added to the solution, which had become almost colourless, and the alcohol was distilled off under reduced pressure; the aqueous solution was cooled, filtered and cautiously neutralised with hydrochloric acid; after standing in ice for a few hours, the white crystalline precipitate was filtered off. Yield 4·0 g. It was recrystallised from dilute alcohol and formed colourless rectangular plates, M.P. 177·5°.

15·4 mg. gave 1·33 mg. N (Micro-Kjeldahl).

Found	N	8·6 %
Calc. for $C_{17}H_{15}O_5N_2$	N	9·0 %

β-[4-(4′-*Methoxyphenoxy*) *phenyl*]-*α-aminopropionic acid*. 2·5 g. 4-(4′-methoxyphenoxy) benzylhydantoin were refluxed for 6½ hours with 30 g. crystalline barium hydroxide and 50 cc. water; the hydantoin was very insoluble in the barium hydroxide solution and was recovered for the most part unchanged; the filtrate was made acid to Congo red with sulphuric acid, filtered from barium sulphate and the filtrate and washings treated with sodium acetate until no free mineral acid was present; on concentrating on the water-bath a minute amount of an amino-acid crystallised out. On recrystallisation

it formed bunches of small needles, which, on heating, darkened at 210° and melted with decomposition at 220–221°.

18·6 mg. gave 0·9 mg. N (Micro-Kjeldahl).
16·7 mg. gave 1·34 cc. moist N at 17° and 754 mm. (Van Slyke).

	Total N	NH_2-N
Found	4·8 %	4·6 %
Calc. for $C_{16}H_{17}O_4N$	4·9 %	4·9 %

On boiling with hydriodic acid it gave a substance having the appearance of desiodo-thyroxine, but the amount was too small for identification. A better method of preparing the desired amino-acid consisted in simultaneous reduction, hydrolysis and demethylation of the 4-(4′-methoxyphenoxy) benzalhydantoin by boiling with hydriodic acid and red phosphorus.

4-(4′-*Hydroxyphenoxy*) *benzylhydantoin*. This compound was formed on boiling 4-(4′-methoxyphenoxy) benzalhydantoin for 1 hour with 10 parts of hydriodic acid (sp.g. 1·7) and 1 part of red phosphorus. Crystallised from very dilute alcohol it formed small colourless cubes, M.P. 245–246°.

15·5 mg. gave 1·38 mg. N (Micro-Kjeldahl).

Found	N 8·9 %
Calc. for $C_{16}H_{16}O_5N_2$	N 9·1 %

β-[4-(4′-*Hydroxyphenoxy*) *phenyl*]-*α-aminopropionic acid*. 2 g. 4-(4′-methoxyphenoxy) benzalhydantoin were boiled for 4 hours with 20 cc. hydriodic acid (sp.g. 1·7) and 2 g. red phosphorus; a further 3 g. red phosphorus was then added and the boiling continued for 5 hours more. The resulting solution was worked up in precisely the same manner as that described under the synthesis by the glycine anhydride method. There was obtained just under 1 g. of an amino-acid having the same solubilities, reactions and crystalline appearance as desiodo-thyroxine. It melted with decomposition at 252–253°.

Analysis. 0·1073 g. gave 0·2587 g. CO_2; 0·0543 g. H_2O.
20·8 mg. gave 1·02 mg. N (Micro-Kjeldahl).
20·0 mg. gave 1·80 cc. moist N at 17° and 754 mm. (Van Slyke).

	C	H	N (total)	N (amino)
Found	65·75	5·6	4·9	5·2
Calc. for $C_{15}H_{15}O_4N$	65·9	5·5	5·1	5·1

The *hydrochloride*, prepared as described above, had M.P. 238–240°, mixed with the hydrochloride of natural desiodo-thyroxine, 237–239°.

0·1263 g. gave 0·0576 g. AgCl.

Found	Cl 11·3 %
Calc. for $C_{15}H_{16}O_4NCl$	Cl 11·5 %

The identity of natural desiodo-thyroxine and the synthetic amino-acid is therefore established.

In conclusion I desire to express my sincere gratitude to Prof. G. Barger, F.R.S., for his continued interest in this work and for much valuable advice.

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XLI. THE INTERMEDIARY METABOLISM OF CARBOHYDRATES. GLYOXALASE CONTENT OF RABBIT'S MUSCLE.

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The mechanism of the production of lactic acid from carbohydrates in the animal body is a problem of fundamental importance, and the view that methylglyoxal may be the immediate precursor of lactic acid received strong support when it was demonstrated that an enzyme capable of converting methylglyoxal into lactic acid with great rapidity is present in animal tissues [Dakin and Dudley, 1913, 1, 2].

This enzyme, glyoxalase, was found in all the tissues examined, except the pancreas, of a variety of animals, as well as in yeast; it is therefore widely distributed, and it was a matter of considerable surprise when in a recent paper Foster [1925] reported its absence from rabbit's muscle, particularly as it had been detected in the muscle of several other animals. This finding involved, of course, a deduction of great moment, for, if glyoxalase be absent from rabbit's muscle, it is extremely unlikely that methylglyoxal may be a precursor of the lactic acid which is undoubtedly formed in quantity by this tissue. Foster suggests, therefore, that muscle may have a specialised carbohydrate metabolism of its own for the production of the lactic acid required in the processes concerned with muscular movement and different from that concerned in the "general metabolism" of muscle and other organs "where lactic acid is merely a stage in the complete combustion of glucose."

The importance of this deduction and the apparently anomalous behaviour of rabbit's muscle prompted a few experiments to test the accuracy of this interesting and unexpected experimental result.

On examination of Foster's actual experiments, in which she investigated the production of *l*-mandelic acid from phenylglyoxal by extracts of rabbit's muscle, using the technique described by Dakin and Dudley [1913, 2, 3] for studying the activity of glyoxalase, it will be seen that in two out of six experiments small but definite negative rotations ($-0{\cdot}21°$ and $-0{\cdot}19°$) are recorded. It is true that they are only about one-tenth of those which are obtained when the equivalent experiment with rabbit's liver is made, but they might be interpreted as indicating a small formation of *l*-mandelic acid by the action of glyoxalase.

It should be borne in mind in this connection that the method of extraction of the mandelic acid from the reaction mixture would undoubtedly result in the concomitant extraction of a certain amount of *d*-lactic acid, and that the rotation of the latter would oppose that of the former, at any rate to a slight extent, and would thus tend to mask the presence of very small amounts of *l*-mandelic acid. The presence of lactic acid might also account for the failure of any small amount of mandelic acid, which may have been present, to crystallise in the characteristic way noted in experiments yielding larger quantities of this substance. It is clear, however, that relatively very little *l*-mandelic acid was formed in Foster's experiments with rabbit's muscle.

In the investigation here reported two experiments were made, by the original technique, exactly comparable with those of Foster, on the same sample of minced rabbit's (thigh) muscle, and the results were in accord with those which she obtained. The residues did not crystallise directly, although they actually contained mandelic acid (see experimental section), and the rotations were $-0{\cdot}15°$ and $-0{\cdot}18°$ respectively, whilst from an equivalent experiment using the animal's liver as the source of the enzyme, a rotation of $-1{\cdot}87°$ and typical crystals of mandelic acid were obtained. The rotations obtained in these experiments on muscle correspond to minute amounts of *l*-mandelic acid, and the further experiments, now to be described, warrant the supposition that they were due to this substance and that a definite, though very weak, glyoxalase action was indicated even under these conditions.

It was shown by Dakin and Dudley [1913, 2] that the action of glyoxalase in a 20 % extract of mixed rabbit's liver and muscle is almost completely inhibited by 0·1 % acetic acid and that this concentration of acid completely destroys the enzyme in less than $3\frac{1}{2}$ hours; and, further, that the conversion of methylglyoxal into lactic acid by glyoxalase ceases when the concentration of lactic acid in the digest reaches 0·085 %. The extracts used by Dakin and Dudley and by Foster were prepared by digesting the minced tissue with five times its weight of water at room temperature for an hour and then straining through muslin. Now minced rabbit's muscle is known rapidly to produce large amounts of lactic acid, as is pointed out by Foster herself. Assuming a potential lactic acid production of about 0·5 %, which is well within the range of yields reported in Foster's paper, the lactic acid content of a 20 % muscle extract might reach about 0·1 %. This, of course, would not all be free lactic acid on account of the buffering effect of the tissue, but it seemed reasonable to suspect that the failure of rabbit's muscle extracts to exhibit vigorous glyoxalase activity might be due, not to actual absence of the enzyme from the tissue, but to its destruction by the acids formed during the mincing and the period of extraction of the tissue with water.

Experiments were therefore made in which fresh rabbit's muscle, after being minced as quickly as possible, was extracted in sodium carbonate solutions of various strengths in order to ensure prompt neutralisation of the acid formed.

The presence of glyoxalase in such extracts was unmistakably demonstrable, rotations of – 1°, due to *l*-mandelic acid formed by its activity, being observed, whilst the acid itself was isolated and identified.

There is no question, then, that rabbit's muscle, in common with other mammalian muscle, contains glyoxalase. The hypothesis that methylglyoxal may be the precursor of lactic acid in carbohydrate metabolism is, therefore, not less valid for rabbit's muscle than for other tissues, and the general findings of Dakin and Dudley concerning the distribution and function of glyoxalase require no modification. Since Foster's experiments failed to reveal definitely the glyoxalase actually present in rabbit's muscle they cannot be regarded as affording any experimental support for the hypothesis that muscle may have alternative metabolic paths for the production of lactic acid from carbohydrate, according to the purpose for which the lactic acid is needed.

The new and interesting point brought to light by Foster's experiments is that rabbit's muscle alone, of all muscle so far examined, fails to yield an active extract to water.

It is probably significant that, with the exception of the fowl, the activity of the glyoxalase of the livers of the animals examined, as judged by the formation of *l*-mandelic acid from phenylglyoxal, has always been considerably greater than that of corresponding amounts of muscle from the same animals. It is possible that a partial destruction of the glyoxalase present in muscle may always take place during simple extraction of the minced tissue with water, though it is surprising, to take the extreme cases, that the same procedure should give a practically inactive extract from rabbit's (thigh) muscle and a very active extract from fowl's (breast) muscle. The latter tissue is stated to contain a relatively large amount of lactacidogen [Lyding, 1921], and Lyding is of the opinion that in the muscle of birds this compound breaks down with greater rapidity *post mortem* than in mammalian muscle; presumably, therefore, lactic and phosphoric acids will be produced in extracts of the minced fowl's muscle as promptly and in quantities at any rate as large as in similar extracts of rabbit's muscle. The glyoxalase of rabbit's muscle may be more sensitive to acid than that of fowl's muscle, or the efficiency of the natural buffering during extraction may be different in the two cases, but the elucidation of this curious difference between the glyoxalases of rabbit's and fowl's muscle must form the subject of a separate investigation. The experiments so far made on the occurrence of glyoxalase in muscle do not bear quantitative interpretation, especially in view of the results reported in this communication, nor have they, as a rule, been made on particular muscles of the animals used. It is worthy of note that Emden and his collaborators [Emden and Adler, 1921; Lyding, 1921] have shown that, in rabbits and fowls, the amount of lactacidogen in the rapidly contractile and readily fatiguable muscle (white muscle) is considerably greater than in the relatively more slowly contractile and difficultly fatiguable muscle (red muscle) of the same animal, whilst the reverse relationship holds in the case of the "residual

phosphoric acid" of these muscles. It seems possible, therefore, that the glyoxalase of such muscles, which is probably intimately involved in the production of lactic acid in these organs, may differ in quantity or properties, and this point must be considered in any more detailed enquiry into the glyoxalase of muscle.

Experimental.

Glyoxalase content of aqueous extract of rabbit's (thigh) muscle.

A rabbit was killed and its thigh muscles were immediately removed, minced and suspended in five times their weight of distilled water. The liver was similarly treated. After occasional stirring for 1½ hours the extracts were strained through cheese-cloth and the following experiments made:

(*a*) and (*b*). To 50 cc. of the muscle extract were added 10 cc. of a 2 % solution of phenylglyoxal, 10 cc. of a suspension of chalk in water and a few drops of toluene.

(*c*). The same additions were made to 50 cc. liver extract.

After incubation at 37° for 22 hours 25 g. solid ammonium sulphate were added to each of the three flasks which were then heated in a boiling water-bath until precipitation of the protein was complete (about 10 minutes). After cooling, 10 cc. syrupy phosphoric acid were added to each of the mixtures which, after standing for 10 minutes, were filtered on a Buchner funnel. The clear filtrates were extracted four times with ether and the ethereal solutions were evaporated.

Experiments (*a*) and (*b*) gave small syrupy residues, whilst (*c*) yielded a typical crystalline residue of mandelic acid. The residues were dissolved in 16 cc. water, clarified by means of a pinch of charcoal, filtered and examined in a 2 dm. polarimeter tube (Hg green light).

The observed rotations were: (*a*) − 0·15°, (*b*) − 0·18°, (*c*) − 1·87°.

Blank experiments from which phenylglyoxal is omitted, usually give very slight positive rotations.

The glyoxalase activity of the liver extract is rather more than 10 times that of the muscle extract.

After polarimetric examination solutions (*a*) and (*b*) were evaporated to dryness on the water-bath, when a crystalline residue was obtained. This was dissolved in water and the solution was shaken four times with ether. On evaporating the ethereal solution to dryness a crystalline residue, weighing 0·1467 g., was obtained, which, after recrystallisation from benzene, melted at 116–118°. This was obviously *r*-mandelic acid. It is evident, therefore, that mandelic acid can be present without necessarily crystallising in the residue obtained by the ordinary technique from experiments with rabbit's muscle.

On account of this rather large quantity of *r*-mandelic acid an experiment was made to determine whether incubation of phenylglyoxal solution with chalk alone resulted in the formation of *r*-mandelic acid. 120 cc. water containing 0·4 g. phenylglyoxal and 10 cc. chalk suspension with 2 cc. of toluene

were incubated at 37° for 23 hours. 50 g. ammonium sulphate were then added, the solution was heated for 10 minutes in a boiling water-bath, cooled, acidified with 20 cc. syrupy phosphoric acid, and filtered. The filtrate was extracted four times with ether from which 0·1054 g. *r*-mandelic acid (M.P. 118°) was obtained. The phenylglyoxal used throughout these experiments was an old commercial specimen and may have contained some mandelic acid. An attempt to determine whether this was the case was made by treating 0·4 g. with aqueous ammonia, which combines with phenylglyoxal to form a yellow, insoluble compound. After acidification and filtration 0·0664 g. mandelic acid was extracted from the filtrate.

It would be necessary to perform this experiment with pure phenylglyoxal to be certain whether no mandelic acid at all is formed by the action of ammonia, but apparently a small amount of inactive mandelic acid may be formed by the digestion of phenylglyoxal with a suspension of calcium carbonate. It is worthy of note in passing that the amount of mandelic acid obtained from the rabbit's muscle experiments was definitely larger than that from the simple digestion of an equal amount of the glyoxal with chalk suspension, but whether any of the former resulted from actual enzyme activity must remain for the present undecided [cf. Dakin and Dudley, 1913, 2].

Glyoxalase content of alkaline suspensions and extracts of rabbit's (thigh) muscle.

A rabbit was killed and its thigh muscles were removed and minced as quickly as possible.

The following experiments were made:

(*d*) 20 g. of the minced muscle were suspended in 100 cc. 0·1 % Na_2CO_3; 10 cc. of a 2 % solution of phenylglyoxal, 10 cc. of a suspension of chalk in water and 0·3 cc. toluene were immediately added.

(*e*) As in (*d*), except that the minced tissue was suspended in 0·25 % Na_2CO_3.

(*f*) As in (*d*), except that the minced tissue was suspended in 0·5 % Na_2CO_3.

The flasks containing these mixtures were then placed in the hot room at 37°.

Two extracts were made, (1) by digesting a portion of the minced muscle with five times its weight of 0·1 % Na_2CO_3 solution and (2) by digesting a portion with five times its weight of 0·5 % Na_2CO_3 for 1 hour, after which the fluids were strained through muslin and the following experiments made:

(*g*) To 50 cc. of extract (1) phenylglyoxal, chalk and toluene were added as in the previous experiments.

(*h*) To 50 cc. of extract (2) phenylglyoxal, chalk and toluene were added as in the previous experiments.

The flasks containing these mixtures were then incubated at 37°. After 20 hours' incubation they were worked up in the manner described above.

The residues in all experiments, after removal of the ether, were syrupy, containing scanty amounts of crystalline material. They were each dissolved in 16 cc. water, clarified with a pinch of charcoal and filtered. The rotations of these solutions were examined in a 2 dm. tube with Hg green light.

The following rotations were observed:

(d) $-1{\cdot}01°$, (e) $-0{\cdot}94°$, (f) $-0{\cdot}59°$, (g) $-0{\cdot}40°$, (h) $-1{\cdot}06°$.

The solutions, after polarimetric examination, were combined and evaporated to dryness on the water bath. A crystalline residue of mandelic acid resulted. This was dissolved in water and the solution extracted with ether. The mandelic acid, after removal of the ether, was recrystallised twice from benzene. 0·1770 g. of the recrystallised acid, dissolved in 16 cc. water and examined in a 2 dm. tube with Hg green light, displayed a rotation of $-1{\cdot}97°$.

It has been shown that a certain amount of r-mandelic acid was obtained by simple incubation of the phenylglyoxal with a suspension of chalk, and it is well known that alkalis readily convert glyoxals into the corresponding hydroxy-acids.

Sir F. G. Hopkins, to whom I am very grateful for his friendly criticism, suggested that it was conceivable that the l-mandelic acid found in these experiments might have arisen, not from the action of a glyoxalase on the phenylglyoxal, but by virtue of a preferential oxidation of the *dextro*-form in racemic acid produced by the direct action of the alkali on the phenylglyoxal, at any rate in the most strongly alkaline of the above mixtures. The following experiments were accordingly made to investigate this possibility.

Comparative effects of suspensions and extracts of rabbit's muscle in 0·1 % and 0·5 % Na_2CO_3 on phenylglyoxal and r-mandelic acid.

Suspensions. The thigh muscles of a freshly killed rabbit were used.

(i) Exactly as (d).

(j) Exactly as (d), except that, instead of 0·2 g. phenylglyoxal, 0·2 g. r-mandelic acid was added as neutral Na salt in 15 cc. water.

(k) Exactly as (f).

(l) Exactly as (f), except that, instead of 0·2 g. phenylglyoxal, 0·2 g. r-mandelic acid was added as neutral Na salt in 15 cc. water.

The extracts were incubated at 37° for 20 hours.

Extracts. The thigh muscles of another rabbit were used.

(m) Exactly as (g) except that 100 cc. extract was used.

(n) Exactly as (g), except that 100 cc. extract was used and 0·2 g. r-mandelic acid was added as neutral Na salt in 15 cc. water.

(o) Exactly as in (h), except that 100 cc. extract was used.

(p) Exactly as in (h), except that 100 cc. extract was used and 0·2 g. r-mandelic acid was added as neutral Na salt in 15 cc. water.

The mixtures were incubated at 37° for 23 hours.

The experiments were worked up in the way previously described. The combined ether extracts in each experiment were washed once with a small

quantity of water before the removal of the ether. Mandelic acid then crystallised in each residue in the normal manner.

The rotations of the residues, dissolved in 16 cc. water and observed in a 2 dm. tube in Hg green light, were as follows:

(*i*) $-1{\cdot}03°$; (*j*) $+0{\cdot}01°$; (*k*) $-0{\cdot}45°$; (*l*) $+0{\cdot}05°$;
(*m*) $-0{\cdot}57°$; (*n*) $0°$; (*o*) $-0{\cdot}85°$; (*p*) $0°$.

Thus it is seen that no *l*-mandelic acid was produced in those experiments where *r*-mandelic acid was added, whilst it was present in every case where phenylglyoxal was the substrate. Under these conditions, therefore, there is no preferential oxidation of one optical isomeride of mandelic acid by alkaline muscle extracts or tissue suspensions.

After polarimetric examination the solutions of active mandelic acid from the experiments with 0·1 % Na_2CO_3 (*i* and *m*) were combined and evaporated to dryness and those from the experiments with 0·5 % Na_2CO_3 (*k* and *o*) were similarly treated. The residues were examined to ascertain whether there was any significant difference in the proportion of *l*-acid in them. That from the 0·1 % Na_2CO_3 experiments weighed 0·2378 g. and had a rotation of $-1{\cdot}24°$ when dissolved in 16 cc. water and examined in a 2 dm. tube with Na yellow light, indicating the presence of 19·6 % *l*-mandelic acid. The residue from the 0·5 % Na_2CO_3 experiments weighed 0·1407 g. and, under the same conditions, had a rotation of $-0{\cdot}91°$, indicating the presence of 24·3 % *l*-mandelic acid.

It appears, therefore, that under the conditions of these experiments the more strongly alkaline solutions had no effect in increasing the amount of *r*-mandelic acid, which might have been expected to be formed by direct chemical action, relative to the *l*-acid arising undoubtedly from enzyme action.

Summary.

Foster's [1925] observation, that rabbit's muscle, unlike all other muscle so far examined, fails to yield an aqueous extract in which the enzyme glyoxalase is clearly demonstrable, is confirmed. Her conclusion, however, that glyoxalase is absent from this tissue is shown to be erroneous, since it can be detected in alkaline extracts.

The hypothesis that in muscle there may be two distinct metabolic paths for the production of lactic acid from carbohydrate is thus deprived of experimental support.

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XLII. NOTE ON THE VARIATION IN THE END-PRODUCTS OF BACTERIAL FERMENTATION RESULTING FROM INCREASED COMBINED OXYGEN IN THE SUBSTRATE.

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The work of Harden [1901] and of Grey [1919, 1, 2] and others has shown that the end products of the bacterial degradation of glucose and of mannitol are qualitatively the same, but differ considerably in the relative quantity of the substances formed. Thus, under similar conditions of fermentation, *B. coli communis* produces twice as much alcohol from mannitol as it does from glucose, but much smaller amounts of lactic acid from the former than from the latter. With a view to obtaining further information as to the effect of the composition of the substrate on the products of bacterial fermentation it was thought that it would be interesting to investigate the more oxygenated derivatives of glucose. Some information with regard to the intermediate region between complete anaerobiosis and aerobiosis might also be obtained by thus presenting to the organism progressively more oxygen already bound in the substrate molecule.

The result which was anticipated has been demonstrated, namely that increasingly oxygenated end-products are obtained from the anaerobic fermentation of increasingly oxygenated substrates, and some indirect support has been given to the view that certain portions of the substrate molecule provide the molecular framework of particular end-products. These results, however, cannot be accepted as conclusive until the experiments described in this note, the greater number of which have been conducted in peptone solutions, have been repeated with synthetic media of known composition, an opportunity for which has not yet arisen.

The action of *B. coli communis* (Escherich, No. 86 National Type Collection) under anaerobic conditions on a series of progressively more oxidised compounds related to glucose has been investigated qualitatively and quantitatively. A few similar experiments have also been carried out with *B. lactis aerogenes*. Below are the formulae of the substrates used arranged in order of their oxygen content, the most oxygenated compound being on the right.

Mannitol	Glucose	Gluconic acid	Glycuronic acid	Saccharic acid
CH_2OH	CHO	$COOH$	CHO	$COOH$
$\vert$	$\vert$	$\vert$	$\vert$	$\vert$
$(CHOH)_4$	$(CHOH)_4$	$(CHOH)_4$	$(CHOH)_4$	$(CHOH)_4$
$\vert$	$\vert$	$\vert$	$\vert$	$\vert$
CH_2OH	CH_2OH	CH_2OH	$COOH$	$COOH$
$C_6H_{14}O_6$	$C_6H_{12}O_6$	$C_6H_{12}O_7$	$C_6H_{10}O_7$	$C_6H_{10}O_8$

Mannitol, which, strictly speaking, does not come into the series, has not been investigated by the present author, but Grey's figures, obtained under similar conditions, for the action of *B. coli communis* on this substance, are used for purposes of comparison, until figures for sorbitol have been arrived at. The fermentations have all been carried out in the presence of an excess of calcium carbonate. In the case of the acids the neutral calcium salts have been used.

Preparation of gluconic, glycuronic and saccharic acids.

Calcium gluconate was prepared by Kiliani's [1884] method with a few modifications. The method as finally modified is as follows: 400 g. of pure anhydrous glucose are dissolved in 1500 cc. water and shaken mechanically in a strong 2½ litre bottle with 450 g. bromine for 3 hours, during which time the bromine should go completely into solution. The mixture is allowed to stand for 48 hours at room temperature, and then concentrated *in vacuo* at 50° to 550 cc. The hydrobromic acid in a measured portion of this is determined by Volhard's method, and enough lead carbonate added to the reaction mixture to combine with the whole of it. This addition is carried out preferably in a large evaporating basin, the lead carbonate being added in small portions at a time. Finally, the temperature of the whole is raised to boiling point and it is allowed to boil for 3 hours, the volume being maintained by addition of water at 500–600 cc. The excess lead carbonate is then filtered off and washed with a little cold water. Most of the lead bromide crystallises out on standing at 0° for 48 hours. It is filtered off, and washed with cold water, and the washings added to the filtrate. The latter still contains appreciable amounts of bromide.

It is then diluted to 2500 cc. and treated with recently precipitated silver oxide suspended in water until a filtered portion of the reaction mixture gives no precipitate with silver nitrate and nitric acid. The silver bromide is filtered off, and washed, and hydrogen sulphide passed to saturation. The filtrate from the sulphide is evaporated to about 1 litre on the water bath and then boiled with calcium carbonate for 3 or 4 hours to decompose any lactone present. The excess calcium carbonate is filtered off and washed, and the filtrate boiled with two grams of powered respirator charcoal. After filtering and washing, the filtrate is put away for 24 hours, and the crude calcium gluconate filtered off and washed with a little cold water. More crude gluconate may be obtained by concentrating the mother liquors. Yield of dry, crude gluconate, 80 % of theory. On two recrystallisations from hot water the pure salt is obtained.

Air dried at room temperature the salt retains one molecule of water of crystallisation, but if the hydrate is left in the hot room at 37° for 3 days the anhydrous salt is produced.

Analysis. Three times recrystallised calcium gluconate left in hot room at 37° for 3 days.

0·5457 g. gave 0·0709 g. CaO = 9·28 % Ca. Calculated for $(C_6H_{11}O_7)_2$ Ca, Ca = 9·30 %.

Glycuronic acid was prepared by Neuberg's [1900] method from euxanthic acid. The glycuronic anhydride was converted into the calcium salt by heating with the calculated quantity of calcium hydroxide.

Saccharic acid was prepared by the method of Tollens and Gaus [1888] as the acid potassium salt. This is twice recrystallised, and then neutralised with ammonia, cooled and treated with the calculated quantity of 10 % calcium chloride, with constant stirring. Calcium saccharate is filtered off and recrystallised from a large volume of hot water. The crystals contain $4H_2O$, which is lost *in vacuo* at room temperature. The calcium salt is not very soluble in water, the saturated solution containing the following quantities of Ca $(C_6H_8O_8).4H_2O$ in 100 cc.: at 0° 0·030 g., at 17° 0·043 g., at 37·5° 0·086 g., at 100° 0·448 g.

Preliminary experiments.

The possibility that these substrates might be of value in characterising organisms of the coli-typhosus group was tested by inoculating tubes containing 1 % of the neutral calcium salts of gluconic, glycuronic and saccharic acids in 1 % peptone with a loopful of an 18-hour culture of 21 organisms of this group, and observing the acid and gas production. Glucose peptone was used as control. All the organisms which produced acid and gas from glucose did so with the three other substrates in 18 hours. Gas and acid production were greatest in the case of glucose, though gluconic acid was only slightly less actively attacked. Morgan's No. 1 bacillus, *B. paratyphosus A* and *B. enteritidis* gave very little acid when grown in calcium saccharate. The extraordinary ability of micro-organisms to obtain the material and energy necessary for growth and reproduction from the most unlikely sources was exemplified by the active way in which all the other organisms of the colon group attacked saccharic acid, a substance more oxidised than acetic acid. The Voges and Proskauer reaction was positive in the case of *B. cloacae* and *B. lactis aerogenes* in tubes containing either glucose or calcium gluconate, but not in the glycuronate and saccharate tubes.

Quantitative experiments.

1. *The action of* B. coli communis *on the mannitol-saccharic acid series of compounds.*

The fermentations were carried out in the apparatus designed by Harden, Thompson and Young [1910]. The hydrogen evolved was not determined in all the experiments, and where this analysis was not done the gas collecting flask was dispensed with and a simplified apparatus employed in which the CO_2 was absorbed directly into potash. The analyses were made on the lines suggested by Harden [1901] and by Grey [1919, 1] with certain modifications rendered necessary by the nature of the substrate.

Excess of precipitated calcium carbonate was always present throughout the fermentation, and the p_H of the medium kept as constant as possible by frequent shaking. The basal medium used was 1 % "bacto" peptone to which was added in various experiments from 1–2 g. % of the substrate. The fermentations were conducted under anaerobic conditions at 37° and were allowed to proceed in each case until the evolution of gas from the reaction flask had practically ceased. The acids obtained were identified by their qualitative reactions, and in many cases by the calcium oxide content of their calcium salts or the silver content of their silver salts. No substance other than those mentioned was found in recognisable quantity in the fermentation mixtures, this qualitative finding being confirmed on the whole by the carbon balance sheets.

The results given below indicate the percentage of the carbon of the substrate destroyed which appeared in the form of the various products of fermentation. Except in the case of the author's glucose figures, the figures are averages of from two to four experiments.

Table I.

	Substrate employed					
		Glucose				
Products	Mannitol (Grey, 1914)	(Harden, 1901)	(present author)	Gluconic acid (present author)	Glycuronic acid (present author)	Saccharic acid (present author)
Lactic acid	24·6	39·5	44·6	35·1	17·5	5·8
Succinic acid	6·9	5·6	5·0	9·6	14·4	15·5
Acetic acid	7·0 } 13·9	18·8 } 24·4	16·8 } 21·8	23·4 } 33·0	48·2 } 62·6	49·1 } 64·6
Formic acid	7·4	0·6	0·8	0·5	0·6	2·5
CO_2	27·3	17·2	12·4	13·8	5·0	22·0
Alcohol	27·0	13·1	16·1	10·8	2·6	1·0
Total percentage of substrate carbon appearing as carbon of above compounds	100·2	94·8	95·1	93·2	88·3	95·9

In Fig. 1 the changes in the amounts of the various products in proceeding from mannitol to saccharic acid are shown diagrammatically.

Notes on Table I and Figure 1.

Alcohol. The alcohol obtained from mannitol (Grey) (two —CH(OH).CH_2OH groups) is about twice that obtained from glucose or gluconic acid (one —CH(OH).CH_2OH group) and that obtained from glycuronic and saccharic acids is almost nil (no —CH(OH).CH_2OH groups). The presence of a —CH(OH).CH_2OH group in the substrate appears necessary for the production of alcohol. Thompson [1912] working with organisms of the coli group found that using malic or citric acid as substrate only traces of alcohol were produced. It will be noticed that neither acid contains a —CH(OH).CH_2OH group.

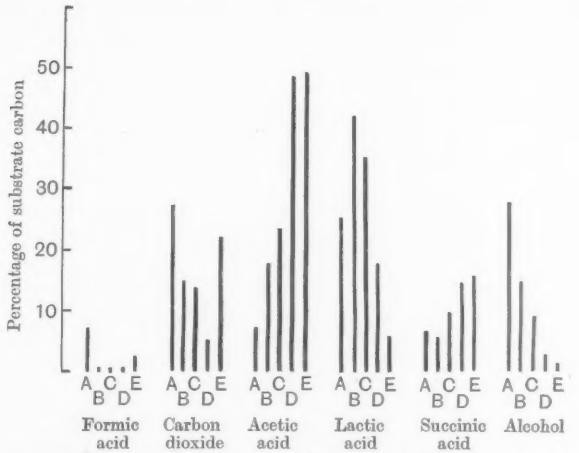

Fig. 1. *A* = amount from mannitol; *B* = amount from glucose; *C* = amount from gluconic acid; *D* = amount from glycuronic acid; *E* = amount from saccharic acid.

Succinic and acetic acids. If succinic acid may be looked upon as two acetic acid molecules joined together [see Grey, 1919, 1] and may be grouped together with it, certain correspondences (which may be fortuitous) appear. Thus the quantity of succinic plus acetic acid produced varies with the number of COOH or potential COOH (*i.e.* CHO) groups in the substance undergoing fermentation. In cases where two COOH (or one COOH and one CHO) groups are present the quantity approaches 66 %, where only one is present it is about 33 %.

Oxalic acid was not found in any of these fermentations.

Methane was not present in recognisable quantity in the gases evolved.

Hydrogen was present in the fermentation gases throughout. Even in the case of saccharic acid, with a relatively large amount of oxygen present in the molecule, a small but quite definite amount of hydrogen was produced.

2. *Action of* B. lactis aerogenes *on mannitol, glucose, gluconic acid and saccharic acid.*

Although *B. lactis aerogenes* is closely related in many of its qualitative reactions to *B. coli communis*, Harden and Walpole [1906] some years ago showed that the end-products of fermentation of glucose and mannitol by this organism, and by *B. coli*, are quantitatively very different. They found that *B. lactis aerogenes* formed, during the fermentation of glucose and mannitol, not only all the end-products of *B. coli* fermentations but also a keto-alcohol, acetylmethylcarbinol, and a glycol, 2 : 3-butylene glycol.

It was decided therefore to examine the chemical action of this organism also on the more oxygenated derivatives of glucose. The methods of fermentation and analysis employed were similar to those used for *B. coli communis*.

The following table shows the results obtained for gluconic acid and saccharic acid, together with those of Harden and Walpole for two other members of the series—glucose and mannitol.

Table II.

Products	Substrate employed (Harden and Walpole) Mannitol	Glucose	Gluconic acid	Saccharic acid
Lactic acid	8·6	5·5	24·1	1·2
Succinic acid	3·2	2·4	2·2	5·4
Acetic acid	2·5	5·1	14·3	50·0
Formic acid	1·5	1·0	2·8	1·5
CO_2	35·5	38·0	24·4	43·1
Alcohol	32·5	17·1	4·8	1·0
Acetylmethylcarbinol	+	+	+	−
Total	83·8	69·1*	72·6	102·2

* The greater proportion of the missing carbon was shown by Harden and Walpole to be present in the form of 2 : 3-butylene glycol.

Notes on Table II.

The experiments reveal the same type of effect as observed with *B. coli*, *i.e.* with increasing oxygen content of the substrate progressive diminution in the alcohol, and increase in the acetic and succinic acids produced. Where no —CH(OH).CH_2OH group is present in the substrate, again no more than traces of alcohol are found. Lactic acid again shows irregularities, reaching a maximum, in the case of *B. lactis aerogenes*, with gluconic acid, whereas with *B. coli* the maximum lactic acid production occurs with glucose.

Acetylmethylcarbinol is not produced from saccharic acid, and is probably, like ethyl alcohol, produced only if there is a terminal CH_2(OH).CH(OH) group present in the fermented molecule. Thompson [1912] found, for example, that *B. cloacae*, which is closely related to *B. lactis aerogenes*, and which produces acetylmethylcarbinol freely from glucose and mannitol, gave no trace of this carbinol when the substrate was malic acid or citric acid, although a straight chain of four carbon atoms is present in both these substances. The present author was unable to detect acetylmethylcarbinol in the products of fermentation of calcium saccharate by *B. cloacae*, but calcium gluconate gave a strong positive Voges and Proskauer reaction.

3. *Stages in the fermentation of calcium gluconate.*

Since the end-products of the fermentation of glucose and of calcium gluconate by *B. coli* were not greatly dissimilar it was decided to investigate the progress of the fermentation of the latter substance to see if the synthetic period of the *B. coli*-glucose fermentation, described by Grey [1919, 2], during which a non-reducing carbohydrate was produced, was also a stage in the fermentation of gluconic acid.

Into each of five flasks, with CO_2 absorbing apparatus attached, were placed 14 g. of calcium gluconate, 7 g. of precipitated chalk and 800 cc. of water. No peptone was used. The flasks were sterilised in the usual way and nitrogen passed through the medium for an hour at 37°. Each was then inoculated with 25 cc. of an emulsion of *B. coli communis* yielding 0·24 g. of dried bacteria per 25 cc. All were placed in the same water-bath at 37°. At the end of 12, 24, 48, 72, 96 hours one flask was removed, and a sample of the medium taken for a bacterial count; the flask was then heated to 70° for half an hour to stop the fermentation, and analyses carried out on the usual lines, using micro-methods wherever possible. The figures obtained are shown in Fig. 2.

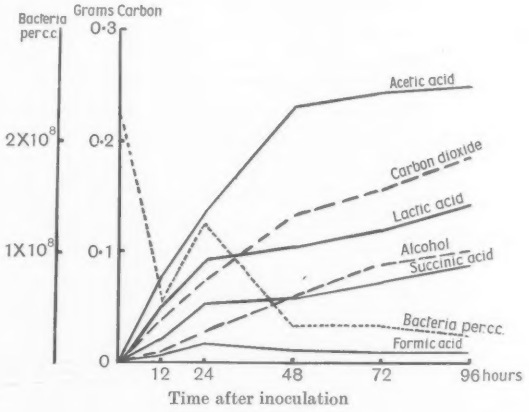

Fig. 2.

The presence of carbohydrate, both reducing and non-reducing, was carefully sought in each flask, but no appreciable amount was produced during the fermentation. Under the conditions of this experiment, with calcium gluconate as substrate, the intermediate production of a non-reducing carbohydrate which occurs in the early stages of the fermentation of glucose by *B. coli*, appears not to take place.

The above experiment also serves to demonstrate the marked difference in the relative proportions of end-products resulting from the fermentation of gluconic acid under varying conditions (peptone present and the culture seeded with a small number of organisms in one case; peptone absent and an emulsion of organisms used in the other) (see Table III). It must be pointed out, however, that, in the 96-hour experiment in absence of peptone, less than one-fifth of the total carbon present in the solution had been broken down, so that the fermentation was still in its early stages.

Table III. *Comparison of products obtained by fermenting calcium gluconate by* B. coli communis *in presence and absence of peptone, shown as percentages of the substrate carbon transformed.*

	Peptone present. Small seeding (fermented till evolution of gas has ceased)	*Peptone absent.* Emulsion of organisms added (after 96 hours' fermentation)
Lactic acid	35·1	17·6
Succinic acid	9·6	11·4
Acetic acid	23·4	33·3
Formic acid	0·5	1·14
CO_2	13·8	25·8
Alcohol	10·8	13·4

The considerable variation in the quantities of fermentation products obtained in the two cases may be due either to the fact that the fermentation was still in progress in the second case, and still far from equilibrium, or that the absolute and relative quantities of these products are dependent on the presence or absence of peptone or the different mode of inoculation. In the case of CO_2 and lactic acid the figures are widely different. It has been assumed that bacteria of the coli-typhosus group grown in a peptone medium containing sugar or other easily fermentable substrate do not attack the peptone to any appreciable extent. The present writer feels that this assumption, particularly in cases where gluconic, glycuronic or saccharic acids are substrates, is open to considerable doubt. Thus there is no question that the organisms increase in quantity during fermentation of glucose in presence of peptone, and the nitrogen required to form the increased quantity of bacterial protein must come from the peptone. When it is remembered that the increased carbon content of the bacteria themselves was not estimated, the amounts of carbon found in the products of fermentation bear in some cases a disturbingly high ratio to the amount of carbon of the substrate consumed. Amino-acids, such as alanine, aspartic and glutamic acids, which are probably liberated in small quantities from protein combination by the enzymes by which the growing organisms provide themselves with nitrogen, are easily capable of slight further change, in presence of the bacteria, to give lactic or succinic acids. It is probable that a proportion of the succinic acid and lactic acid is so derived from the peptone, and until the above experiments are repeated on media which do not contain carbon in addition to that of the substrate, the lactic acid and succinic acid figures, at least, must be regarded with suspicion. It is hoped, later, to repeat this work, using media of known constitution, when it may be found possible to construct more reliable balance sheets, showing the fate of the carbon and hydrogen of the metabolised substrate. With further information as to the heats of combustion of the substances concerned, an energy balance sheet will also become possible. The discussion of the significance of the figures obtained can then be resumed on a far more satisfactory basis.

SUMMARY.

Gluconic, glycuronic and saccharic acids are readily fermented by a large number of organisms of the coli-typhosus group.

Qualitatively, *B. coli communis* and *B. lactis aerogenes* produce from these substances the same group of fermentation products as occur when these organisms act on glucose or mannitol.

Quantitatively, interesting relationships are observed between the chemical composition of the substrate and the quantity of certain of the end-products of fermentation.

The author gratefully acknowledges the hospitality of the Lister Institute during the course of this work and in particular his indebtedness to Prof. Arthur Harden for advice and encouragement.

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XLIII. THE BIOLOGICAL VALUE OF BREAD-PROTEIN.

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I HAVE read with great interest the work of Martin and Robison [1922] on the biological value of various proteins for human nutrition, which I am sorry not to have seen before. Twelve years ago I carried out similar experiments [Hindhede, 1913, 1914] on several persons extending over much longer periods (309 and 126 days); but, whereas I found the protein of bread to be of full value, Martin and Robison have found a value of only 30–35 % as compared with animal protein.

How are these entirely different results to be explained? In my opinion the explanation must be sought in the fact that these investigators did not continue for any prolonged period on a sufficiently low quantity of digestible nitrogen (nitrogen in food – nitrogen in faeces). According to my experience the nitrogen minimum in urine cannot be attained in a few days. Of course on a bread diet it is no easy matter to reduce the nitrogen intake to a low level, whilst maintaining a sufficient calorie value, bread being comparatively rich in nitrogen. By chance we discovered that this was practicable by adding fruit, which accelerates the passage through the intestines, thus reducing the absorption, especially of nitrogenous compounds.

The following examples serve to illustrate this fact.

F. M.* Period III, 1913. 12 days.

One day	Weight g.	Dry matter g.	N g.	Calories
Rye-bread	1000	661	11·71	2817
Margarine	125	108	0·16	984
In food	1125	769	11·87	3801
In faeces	417	96	4·42	480
Absorbed	—	673	7·45	3321
In urine	—	—	7·33	—
Balance	—	—	+0·12	—
Loss, %	—	12·5	37·2	12·6

F. M. Period X, 1913. 12 days.

Rye-bread	542	356	6·72	1523
Margarine	121	104	0·16	953
Prunes, dried	625	338	2·06	1383
Sugar	108	107	—	442
Starch	75	61	0·01	252
In food	1471	966	8·95	4553
In faeces	833	127	5·62	608
Absorbed	—	839	3·33	3945
In urine	—	—	3·62	—
Balance			−0·29	
Loss, %	—	13·1	62·8	13·4

* F. M. = Frederik Madsen. H. M. = Holger Madsen.

F. M. Period XII, 1913. 8 days.

One day	Weight g.	Dry matter g.	N g.	Calories
Rye-bread	550	361	7·09	1549
Margarine	125	108	0·16	984
Strawberries	2500	267	3·40	1083
Sugar	275	272	—	1127
Starch	138	112	0·02	464
In food	3588	1120	10·67	5207
In faeces	875	167	6·85	797
Absorbed	—	953	3·82	4407
In urine	—	—	2·83	—
Balance	—	—	+0·99	—
Loss, %	—	15·10	64·2	15·3

It will be seen from the last period that a net intake of 3·82 g. N was sufficient not only to cover the output of nitrogen in the urine, but even to store 1 g. N per day in the body. But it may be said that probably the protein in the fruit has a high value. Is it possible that the 3·82 g. absorbed N was almost entirely derived from the protein of the strawberries (3·40 g. N) whilst practically all the bread-protein passed out with the faeces? None of the witnesses of the experiments thinks so. The enormous mass of faeces seemed to consist almost solely of strawberry seeds, and there is hardly any doubt that the N of strawberries is chiefly present in the seeds, which pass through the intestines almost unchanged. We tested the digestibility of the protein by treating it for 48 hours in the thermostat with pepsin-HCl and found that there were digested of

Protein in ryebread (unbolted)	76 %
,, prunes	33 %
,, strawberries ...	38 %

Forty-eight hours in the thermostat is very energetic treatment, with which the animal stomach does not seem to be able to compete.

Our previous experiments with potato diets show that there is no limit to the extent to which the digestible nitrogen of food can be reduced by this method. The following example may be given.

F. M. Period V, 1912. 8 days.
Food per day: 2354 g. potatoes, 153 g. margarine.

	Dry matter g.	N g.	Calories
In food	717	5·35	3609
In faeces	16	0·85	82
Absorbed	701	4·50	3527
Loss, %	2·2	15·9	2·3

F. M. Period X, 1912. 8 days.
Food per day: 700 g. potatoes, 75 g. margarine, 2519 g. strawberries, 162 g. starch, 375 g. sugar.

In food	986	5·19	4391
In faeces	128	5·16	624
Absorbed	858	0·03	3767
Loss, %	13·0	99·4	14·2

This method possesses the great advantage of enriching the diet with vitamins and also with alkaline salts which neutralise the acids formed from

the oxidation of the bread-proteins, so that the body is spared the necessity of providing ammonia for this purpose. Moreover it makes the diet considerably more savoury.

The following table gives the average of the N assimilation per day during the various periods.

		F. M.				H. M.			
Period	No. of days	Absorbed N g.	N in urine g.	N-balance g.	Body-weight kg.	Absorbed N g.	N in urine g.	N-balance g.	Body-weight kg.
—	—	—	—	—	78·0	—	—	—	67·0
IV	22	4·00	4·71	−0·71	77·0	3·17	4·05	−0·88	67·5
V	12	4·76	4·89	−0·13	76·0	4·74	5·09	−0·35	66·0
VI	12	5·07	4·89	−0·22	75·5	4·99	4·66	+0·33	68·0
VII	12	4·53	5·11	−0·58	75·5	5·13	4·77	+0·36	67·5
VIII	12	3·70	4·44	−0·74	72·0	**3·55**	**3·37**	+0·18	67·0
IX	12	**3·01**	**3·57**	−0·56	73·0	5·63	4·37	+1·26	67·0
X	12	**3·33**	**3·62**	−0·29	73·0	4·84	4·53	+0·31	67·0
XI	12	**3·64**	**3·50**	+0·14	73·4	**3·16**	**3·55**	−0·39	67·0
XII	6	**3·69**	**3·36**	+0·33	73·6	**4·01**	**2·85**	+1·16	66·5
XIII	8	**3·82**	**2·83**	+0·99	74·0	**3·00**	**3·27**	−0·27	66·0
XIV	6	8·49*	5·86	+2·63	74·0	6·28	5·00	+1·28	66·0
	126								

* In period XIV only bread is consumed. Note that here there is a great surplus of N in the food. This does not agree with Martin and Robison's experiments.

The average of the periods (bold type) in which the amounts of absorbed N were lowest is shown below:

	Per diem		
	Absorbed N	N in urine	Balance
F. M. 50 days	3·45	3·42	+0·03
H. M. 38 „	3·38	3·32	+0·06

From these experiments I conclude that 3·50 g. digestible N, equivalent to 22 g. digestible bread protein is sufficient to keep young, vigorous men in protein balance. How, then, is it to be explained that the two English experimenters could not attain balance on correspondingly low amounts.

Let us look at their figures.

The following table has been compiled from the results given by Martin and Robison, the absorbed N being taken as equal to the N intake minus the total N of the faeces.

		C. J. M.				R. R.			
Period	No. of days	Absorbed N g.	N in urine g.	N-balance g.	Body-weight kg.	Absorbed N g.	N in urine g.	N-balance g.	Body-weight kg.
1	3	3·56	5·62	−2·06	61·5	4·24	5·62	−1·38	57·7
2	3	6·46	7·62	−1·16	61·4	6·59	7·31	−0·72	58·1
3	3	6·62	7·42	−0·80	—	7·66	8·18	−0·52	—
4	3	8·24	7·46	+0·75	61·0	8·30	8·32	−0·02	57·9
5	8	7·90	8·43	−0·53	61·6	8·03	8·93	−0·90	57·4
6	4	9·47	9·23	+0·24	61·4	9·30	9·34	−0·04	57·7
	24								

It will be seen that our experiments extend over a period five times as long as that of the English experimenters. The main difference may be

characterised as follows: when in the course of three days C. J. M. and R. R. do not succeed in obtaining balance on 3·56 or 4·24 g. digestible N, it is taken for granted that balance cannot be obtained on this level and the N in the food is then greatly increased. But as the N in the urine is increased almost proportionately, balance is not attained even by this, and so the intake is further increased. Only at 8–9 g. digestible N is balance attained with C. J. M., but with R. R. balance is not even reached by this. From this it would appear that 50–60 g. digestible bread-protein is required to attain balance, which means that the biological value of bread-protein is only about one-third that of meat-protein (body-protein).

In the Danish experiments the method is reversed. We continue for 22 days with respectively 3·17 and 4·00 g. digestible N, although the N balance is negative. From other experiments we have learned that with a very low level of N intake, the N in the urine does indeed quickly decrease until balance is nearly attained, but months may pass before the last trace of stored N disappears. At the same time we have learned that such a prolonged negative balance is of no consequence whatever as regards the state of health and the capacity for work. In this connection it may be mentioned that a negative balance continued for a prolonged period may prove an excellent cure for a number of chronic diseases[1].

F. M. loses weight during the first months (22. ii. 20. v.), but that is always the case with F. M. during the busy spring time, when, in his capacity as a skilled gardener, he does 6 to 7 hours' hard garden work a day, besides his 7 hours' work at the laboratory. The testimony of one of his employers provides abundant evidence of Madsen's extraordinary vitality and his capacity for long continued labour of the heaviest description whilst on this diet.

During the last 50 days, when the N in his food is most reduced, he does not lose weight, but on the contrary, gains a little. With H. M., the other subject of experiment, the weight remains almost constant. It may be added that he competed in a long-distance walking match and covered 264 miles in 99·35 hours (74·33 hours for walking, 25·02 for rest and sleep), finishing fourth out of nine competitors.

The only way to reduce the N secretion in urine is by a strong reduction of the N intake. Balance is, curiously enough, not infrequently most easily obtained by reducing the N in the food very far—to a certain limit, of course. Only then is the removal effected of the last remnant of stored N which tends to disturb the results.

The English authors use complicated formulae in the calculation of the biological value of the various proteins; but even the most elaborate formulae are to no purpose if the figures put into them are not correct. I find no occasion to go into these formulae. I content myself by concluding that when two young, vigorous men can maintain balance on 22 g. digestible bread-

[1] Cf. Dr Sandoz [1907, p. 219]. "Plus l'histolyse serai intense, plus la transformation qui doit conduire à la guérison serai rapide et complète."

protein, the bread-protein must have essentially the same value as protein in meat and milk. I do not, therefore, consider it correct that the biological value of protein in whole wheat is only 31–35 %. And this scepticism as to the method seems to me to be supported by the fact that, by the same method, Prof. Martin and Dr Robison found that milk-protein has a biological value of only 51 %. Here they are also at variance with the results of other investigators.

Of course there is the difference that Martin and Robison used wheat-bread, whilst we used rye-bread, but according to our other experiments with wheat and rye it seems to me impossible to believe that wheat-protein should be so inferior to rye-protein.

Not till Prof. Martin and Dr Robison have, for a couple of months, tried a diet containing only about 3·50 g. digestible bread-N besides sufficient calories, vitamins and salts—not till then have they furnished any proof of the small value of bread-protein.

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XLIV. THE EFFECT OF IRRADIATION OF THE ENVIRONMENT WITH ULTRA-VIOLET LIGHT UPON THE GROWTH AND CALCIFICATION OF RATS, FED ON A DIET DEFICIENT IN FAT-SOLUBLE VITAMINS. THE PART PLAYED BY IRRADIATED SAWDUST, II

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Introduction.

In a paper published in 1924 [Hume and Smith, 1924], the writers showed that when young rats, fed on a diet deficient in fat-soluble vitamins, are kept in glass jars containing sawdust which has been irradiated with the mercury vapour quartz lamp, they grow and their bones calcify, as is the case with rats which are irradiated directly. The present work was then undertaken to ascertain in what way the sawdust acts, whether the rats have to be in actual contact with it, so that they can consume some of it, which an examination of their faeces shows that they do when they have the opportunity, or whether direct contact is not necessary in order that the effect shall be produced. If the former alternative be the correct one, the work falls into line with that of Steenbock and Black [1924] and of Hess and Weinstock [1924, 1], who showed that oils, inactive in preventing rickets in rats, when fed to them, can be rendered active by irradiation with ultra-violet light. Since then evidence with regard to the capacity of substances, including cholesterol [Drummond *et al.*, 1925; Hess *et al.*, 1925] and phytosterol [Hess *et al.*, 1925], to be activated has accumulated.

On the other hand, though it seemed probable that direct contact between the rat and the irradiated sawdust was necessary in order that the effect might be obtained, there existed a certain amount of evidence showing that irradiated bodies can exercise an action upon a photographic plate, and that they therefore give off some sort of radiation or emanation. Kugelmass and McQuarrie [1924], for example, published experiments which seemed to show that irradiated bodies give off something which is able to fog a photographic plate, even through a transparent quartz screen. Papers published a good many years ago by Russell [1905] showed that many substances, and the wood of conifers in particular, after exposure to a strong light, give off an

emanation which acts strongly on a photographic plate and which he proved was a vapour and not a radiation.

Nelson and Steenbock [1925] found that rats living on wire screens, soiled with their urine and faeces, receive powerful protection from rickets if the screens are irradiated and if the rats are in direct contact with them, so that irradiated excreta are conveyed by the rat's paws to its mouth. But if the screens are suspended 6 inches above the rats' heads, where the animals cannot touch them, no anti-rachitic effect can be demonstrated.

The present experiments were therefore devised to test firstly whether anti-rachitic radiations of any kind are given off from irradiated sawdust, and secondly whether any volatile substance having anti-rachitic potency is given off. The rats in these experiments were separated from the irradiated sawdust by a transparent quartz plate and by wire grids respectively. At the same time the possibility that emanations or radiations, capable of acting on a photographic plate, are given off by irradiated sawdust was examined by Dr N. S. Lucas, who concluded [1926] that nothing is given off, which can pass a transparent quartz screen sealed with airtight joints, but that a vapour is given off which can act at short range on a photographic plate. Ultra-violet radiations are not given off, but some volatile substance is; this substance Lucas held to be hydrogen peroxide, as did Russell, or else ozone.

Technique.

The rats used were all white or black and white, of about 50 g. in weight, and were bred in the Lister Institute. Rats belonging to the same litter were distributed equally, as far as possible, among the various experimental groups to be compared.

The diet used was deficient only in fat-soluble vitamins; full details are set out in the writers' note in the current number of this journal and in former papers [Hume and Smith, 1926].

A mercury vapour quartz lamp, of the Hewittic Electric Co., with straight burner, was used. All irradiations were carried out six times a week. Rats irradiated directly were treated for 10 minutes at a distance of 50 cm. Irradiation of sawdust and of cotton wool in the various experiments took place at distances of from 30–70 cm. and for 10–30 minutes.

The rats were kept singly in cylindrical 8-litre glass jars, except those which were separated from the irradiated sawdust by a quartz plate. The latter were housed in specially constructed, rectangular glass vessels of smaller size.

The sawdust used was ordinary pine sawdust from a sawmill. It was changed every time it was irradiated, so that particles of food or excreta were not irradiated.

The experiments were continued as long as the rats showed any growth, *i.e.* from 10–16 weeks; they were then killed and the bones of the hind legs were cleaned and dried, and calcium estimations were made by the method of Aron and Sebauer [1910].

Experimental.

Four experimental groups were arranged, as follows:

Group 1 consisted of 14 rats, which inhabited glass jars with sawdust at the bottom, but the sawdust was not irradiated.

Group 2 consisted of 14 rats, which inhabited glass jars with irradiated sawdust at the bottom.

Group 3 contained 13 rats, which inhabited glass jars with irradiated sawdust at the bottom, but the rats were separated from the sawdust by a wire grid, of tinned iron wire, 10 mesh. The distance between the rats and the sawdust was not more than an inch. The grids were never irradiated.

Group 4 comprised only 4 rats. Each rat inhabited a square glass box, into which, at about an inch from the bottom, a transparent quartz plate was fixed so as to be airtight. The quartz plate, 0·2 cm. thick, formed the floor on which the rats lived; underneath, at a distance of about an inch, was a layer of irradiated sawdust. The excreta were cleaned up and a small piece of cotton wool was given towards evening, as bedding, otherwise the rats became cold and suffered.

Table I. *Total growth during period of experiment and percentage of calcium in the dry bones of rats placed on a diet deficient in fat-soluble vitamins and treated in various ways with irradiated and non-irradiated sawdust.*

(For the growth the averages for males and females are given separately; for the calcification all the animals in each group are averaged together. Groups 5–7 are comparable with one another, but not with groups 1–4.)

Treatment		Total increase in weight in period of exp. Average g.	No. of animals averaged	% Calcium in dry bones. Average
Group 1.	Rats in jars on sawdust not irradiated	♂ 66 ♀ 52	8 6	18·2
Group 2.	Rats in jars on irradiated sawdust	♂ 92 ♀ 66	8 6	19·4
Group 3.	Rats in jars on wire grid over irradiated sawdust	♂ 91 ♀ 71	9 4	19·3
Group 4.	Rats separated from irradiated sawdust by quartz plate	♂ 58 ♀ 57	2 2	18·0
Group 5.	Rats in jars on wire grid over sawdust not irradiated	♂ 43	3	17·2
Group 6.	Rats in jars on wire grid over irradiated sawdust	♂ 53	3	18·5
Group 7.	Rats in jars on wire grid over non-irradiated sawdust, themselves irradiated directly	♂ 78	2	20·3

In Table I, groups 1–4, the average growth made by the male and by the female rats in each group, together with the average percentages of calcium in the bones of all the rats in each group, are set out. The rats of the control group (1), on non-irradiated sawdust and the rats separated from irradiated sawdust by a quartz plate (group 4), give figures which both for growth and calcification are very near to one another, the result of group 4 must therefore

be taken to be negative. Groups 2 and 3, on the other hand, containing respectively rats in contact with irradiated sawdust and rats only separated from irradiated sawdust by a wire grid, also give results which are very close together, and which both for growth and calcification are definitely, though not very greatly, higher than those of the controls. The results therefore indicate that rats living on a wire grid, adjacent to irradiated sawdust, are subject to some influence, which promotes growth and calcification to a greater degree than occurs with control animals, living in direct contact with sawdust, which has not been irradiated. It was observed in the course of the foregoing experiments that the rats living on the wire grids consumed much more of their own faeces than did those of any of the other groups. This was recognised as a possible source of error. Steenbock, Sell and Nelson [1923], and Dutcher and Francis [1924] have observed that rats on a diet deficient in vitamin B can resist the deficiency much longer, if they have access to their own faeces than if they have not. A fresh set of experiments was therefore set up, in which the controls were arranged somewhat differently.

Three experimental groups were made, in all of which the rats inhabited a glass jar, with sawdust in the bottom, over which was placed a wire grid, as already described.

Group 5 contained 3 controls, which lived on wire trays over non-irradiated sawdust, instead of in direct contact with it, as did the previous set of controls (group 1).

Group 6 was the same as group 5, but the sawdust below the wire grid was irradiated.

Group 7. In group 7 two rats living on wire trays, over non-irradiated sawdust, were themselves irradiated directly.

In Table I, groups 5–7, the results of the three last experiments are set out in the same way as was done for groups 1–4. The rats irradiated directly show a marked superiority, both in growth and calcification, over either of the other groups. The rats on the wire grids over irradiated sawdust, though markedly inferior to the rats irradiated directly, still show a small superiority, both in growth and calcification, to the rats on wire grids over non-irradiated sawdust.

In the course of the above investigation experiments were also made in which rats were kept in contact with (1) sawdust irradiated in a shallow glass tray, instead of at the bottom of a deep jar; (2) sawdust allowed to stand for 2 hours after irradiation before the rats had access to it; and (3) irradiated cotton wool. The percentage of calcium in the bones for a corresponding group of control rats on non-irradiated sawdust was 18·9, and for a corresponding group of rats on irradiated sawdust 20·3; when the sawdust was irradiated in a shallow glass tray it was 20·7; when the sawdust was allowed to stand for 2 hours it was 20·5; when cotton wool was irradiated it was 18·4. The first two experiments give a clear positive result, the activity of the irradiated sawdust is in no way diminished, either by standing for 2 hours, or by irradia-

tion in a shallow glass dish and subsequent removal uncovered to another room. Irradiated cotton wool, on the other hand, is entirely negative, although it was freely eaten by the rats.

Summary and Conclusions.

The clearly negative result obtained when a quartz plate was interposed between the rats and the irradiated sawdust disposes of the possibility that the sawdust exercises its action by means of any secondary radiations which it gives off and which can penetrate quartz. This result agrees with that of Lucas [1926], who found that irradiated sawdust does not give off anything which is able to fog a photographic plate through a transparent quartz screen.

The other results which the writers have obtained are, however, not so easy of interpretation. Even when the experiments were all made under conditions equally favourable to the consumption of their own faeces by the rats, and thereby favourable to a conservation of vitamin reserves, rats living on wire grids over irradiated sawdust still grew and their bones calcified somewhat better than was the case with rats on wire grids over sawdust which had not been irradiated. The effect, however, was small and not sufficient to justify the conclusion that irradiated sawdust gives off a volatile substance, which is able to promote growth and calcification in rats. The only vapour which Lucas could detect coming from irradiated sawdust was hydrogen peroxide or some other peroxide, and Hess [Hess, 1924; Hess and Weinstock, 1924, 2] found that neither hydrogen peroxide nor ozonised water, fed to rats, had any anti-rachitic action. The only conclusion for which the evidence is satisfactory is that irradiated sawdust exercises a beneficial action on growth and calcification when consumed by the rats.

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XLV. A NOTE ON THE PRODUCTION BY IRRADIATION WITH ULTRA-VIOLET LIGHT OF ANTI-RACHITIC PROPERTIES IN STEROLS DERIVED FROM THE SMALL SIAK ILLIPE NUT (*PALAQUIUM BURCKII*).

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It has been shown separately by Drummond [1925] and by Hess [1925], together with their co-workers, that cholesterol, when irradiated with ultra-violet light, becomes activated, so that it is able to promote calcification and temporary growth in young rats fed on a diet deficient in fat-soluble vitamins. The same observation was made by Hess for phytosterol, prepared from cotton-seed oil.

A supply of mixed sterols, derived from quite a different source, *i.e.* the fat of the small Siak Illipe nut (*Palaquium Burckii*), was placed at the writers' disposal by the kindness of Dr C. W. Moore, and it seemed of interest to test whether these sterols possessed the capacity for activation, which has already been demonstrated for cholesterol and phytosterol from other sources.

Preparation of the sterols.

The sterols were prepared in the following manner. The unsaponifiable matter was separated from a quantity of the fat and formed a semicrystalline mass, most of which dissolved on extraction with boiling acetone and was redeposited in the cold, in the form of small colourless needles, melting indefinitely from 170–180°. The recrystallisation was carried out three times, but the substance so obtained still remained a mixture. It is upon this mixture that the tests about to be described were made. Further experiment showed that from the mixture an acetyl derivative could be obtained, which yielded one of the higher sterols in a pure condition. This sterol, which was designated *palasterol*, resembles in all respects androsterol, isolated from *Apocynum androsaemifolium* [Moore, 1909], and lupeol, derived from *Zanthoxylum macrophyllum* [Goodson, 1921]. Androsterol and lupeol are believed to be identical.

Technique.

For the test young rats were used which had been placed on a diet deficient in fat-soluble vitamins at a weight of about 40 g., and which had been on the diet for 33 days and were only growing very slowly. When an anti-rachitic stimulus of any kind, whether light or food, is applied to such rats, they begin to grow for a time at a much faster rate, though they finally decline and die. Subsequent investigation of their bones shows that they are more highly calcified than would be the case with untreated controls.

The diet used was as follows:

Inactivated caseinogen	300 g.	Marmite	75 g.
Starch	750 ,,	Lemon juice	75 cc.
Hardened cotton-seed oil	225 ,,	Distilled water	300 ,,
Salt mixture	75 ,,		

The salt mixture was as follows:

Sodium chloride ...	51·9 parts.	Calcium phosphate	162·0 parts.
Magnesium sulphate ...	164·0 ,,	Calcium lactate ...	390·0 ,,
Sodium dihydrogen phosphate	104·1 ,,	Ferric citrate ...	35·4 ,,
Dipotassium hydrogen phosphate	286·2 ,,		

Black and white rats bred in the Lister Institute were used.

The dose of sterols given was 0·005 g. daily, administered six times a week. The dose was fed embedded in a very small pellet of white bread and hardened cotton-seed oil. Control rats which did not receive the dose received the pellet.

The mixture of sterols, in the form of a white powder, was irradiated by weighing out the dose for two days, spreading the powder as thinly as possible, dry, upon a glass plate covered with paper, and exposing it for 30 minutes, at a distance of 14 inches, to a mercury vapour quartz lamp. The lamp was a powerful one, having only been in use for a few months. It was an Ulviarc burner, type Y2, of the Hewittic Electric Co.; on a circuit of 140 volts it was using a current of 3·5 amperes.

For the direct irradiation of rats to be used as controls a similar lamp was used, which had been in use for over 18 months and which was therefore proportionately less powerful. The rats were exposed for 10 minutes daily, six times a week, at a distance of 50 cm.

At the end of the experiment the leg bones of each rat were dissected out, the water content was determined, the fat was extracted with boiling ether and alcohol and the bones were ashed.

Experimental.

Three groups of animals were used for the experiment, one group of rats receiving irradiated sterols, one of rats receiving non-irradiated sterols and one of control rats irradiated directly. Each group contained three animals, and all belonged to the same litter. The accompanying Table I shows the result. The rats receiving the irradiated sterols increased in weight by 22–31 g. in the period of the experiment; those irradiated directly by 20–27 g., while

Table I. *Data of the increase in weight and composition of the bones of rats on a diet deficient in fat-soluble vitamins.*

Treatment	No. of Rat	Sex	Weight at beginning of treatment g.	Max. weight during treatment g.	Increase in weight g.	Duration of treatment days	% H_2O in fresh bone	% fat in fresh bone	% ash in wet bone	% ash in dry bone	% ash in fat ext. bone
Direct irradiation from 33rd day	213	♀	51	78	27	37	42·6	8·1	27·6	48·2	56·2
	215	♀	50	71	21	34	45·4	10·4	23·8	43·6	53·9
	216	♀	51	71	20	37	47·0	5·5	25·2	47·5	52·9
0·005 g. irradiated sterols daily from 33rd day	217	♀	47	78	31	36	41·8	12·0	25·4	43·6	55·0
	219	♀	49	71	22	34	46·0	9·8	23·4	43·4	53·1
	220	♂	63	92	29	35	43·8	11·1	24·4	43·5	54·3
0·005 g. non-irradiated sterols daily from 33rd day	214	♀	54	60	6	24	51·9	5·2	21·5	44·8	50·2
	218	♀	51	57	6	37	45·6	12·4	21·4	39·4	51·2
	221	♂	57	69	12	34	59·0	0·7	20·4	50·0	50·9

Rat 221 lost 25 % of weight before the end of the experiment and rat 214 about 15 %, with the result that the fat in the bones was largely replaced by water, an occurrence which makes the percentage of ash in the dry bones inordinately high. Rat 214 succumbed to a vitamin A deficiency after 57 days on the deficient diet.

those which received non-irradiated sterols only increased by 6–12 g. The ash percentages corresponded; those of the rats irradiated directly and of the rats which received irradiated sterols were considerably higher than those of the rats which received the non-irradiated material. The mixed sterols derived from the Siak Illipe nut are, therefore, capable of becoming potent as an anti-rachitic when they are irradiated with ultra-violet light, in the same way as cholesterol and phytosterol.

All the rats in the experiment showed marked symptoms of xerophthalmia and signs of decline, due to deprivation of vitamin A, at about the 50th day on the deficient diet. The onset of these symptoms was entirely comparable in all the three groups, indicating that the sterols contained no appreciable quantity of vitamin A, either before or after irradiation.

Summary.

1. A mixture of sterols, from the fat of the small Siak Illipe nut, was irradiated with ultra-violet light, whereby the power of promoting growth and calcification in young rats, on a diet deficient in fat-soluble vitamins, was imparted to the sterols.

2. The mixture of sterols contained no appreciable quantity of vitamin A, either before or after irradiation.

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XLVI. THE CARBOHYDRATE AND FAT METABOLISM OF YEAST.

PART III. THE NATURE OF THE INTERMEDIATE STAGES.

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Various hypotheses have been put forward to explain the steps by which carbohydrate is converted into fatty acids in the living organism. No direct evidence has hitherto been available from the study of the organism itself, and the theories which have been formulated have been based, on the one hand, on a study of laboratory methods of formation of fatty acids, starting from materials which are believed to occur as decomposition products of carbohydrate in the organism; and, on the other hand, on the nature of the fatty acids known to occur in the natural fats. Many years ago Emil Fischer suggested that the carbohydrate molecules directly condense with one another, joining together by the terminal carbon atoms and thus forming straight chains of carbon atoms from which by subsequent oxidation and reduction fatty acids may be formed. Two circumstances have prevented this hypothesis from finding general acceptance. In the first place we are quite unable to make the hexose molecules condense in this way in the laboratory; in the next place the fats which occur naturally contain all the even-numbered carbon atoms from two to twenty and if these indeed be formed by a process of synthesis it is extremely difficult to account for their formation by the method proposed by Fischer. E. F. Armstrong [1924] has suggested that perhaps the unit attacked is the C_{18} unit, the nucleus of most polysaccharides consisting of three C_6 units united through oxygen atoms. Magnus Levy [1902] proposed acetaldehyde as the intermediate substance which could be derived from sugar and which by undergoing the aldol condensation might build up successive acids containing even numbers of carbon atoms linked together in straight chains. Attempts to synthesise the acids by this method in the laboratory lead however to the formation of acids containing not straight but branched chains. Raper [1907] showed, however, that the aldol molecule will condense with itself to form a straight chain of eight carbon atoms and one of us [Smedley, 1911] showed that the same holds good for crotonaldehyde. To explain the formation of straight chains increasing by two carbon atoms

it was suggested by Smedley and Lubrzynska [1913] that pyruvic acid was the required intermediate and they showed that *in vitro* straight chains of even-numbered carbon atoms can be built up from pyruvic acid, in a series increasing by two carbon atoms. More recently, as the result of investigations on yeast, Lindner [1921] has put forward the view that alcohol may condense with itself in the living organism forming straight chain compounds which by subsequent oxidation form the series of even-numbered fatty acids. This hypothesis is built up on the observation that when yeast is incubated in a solution of ethyl alcohol with a free supply of oxygen, an increased storage of fat takes place in the yeast.

For some years past we have been investigating the action of yeast when incubated in solutions of various simple carbon compounds with the object of throwing some light on this subject. Yeast, if given sufficient oxygen, will utilise various simple carbon compounds and form fat and carbohydrate from them, whilst other closely related substances appear to be entirely unassimilated, and we hoped that some information might be gained from a classification of these substances. The experiments can be carried out quantitatively and the complete balance of the carbon worked out, under a variety of conditions. We made therefore a systematic investigation, choosing substances containing two, three and four carbon atoms respectively, and comparing their effect on the storage of fat and carbohydrate by yeast.

Method of Experiment.

This was similar to that already described by us in an earlier paper [1923]. A quantity of yeast was incubated for 22 hours in the solution to be tested, the medium being well oxygenated throughout the whole period. The yeast was then filtered, weighed and examined as previously described. After the yeast had been hydrolysed with normal hydrochloric acid, the residue was filtered, well washed, dried and extracted with ether; the reducing sugar present was determined in the filtrate by Bertrand's method and calculated as glucose. The ether-soluble substance was dried *in vacuo* to constant weight, hydrolysed with alcoholic potash ($N/2$) and the saponification value determined: the soap solution was extracted with ether three times to remove unsaponifiable matter, and the ether solution of the latter washed with dilute alkali and water. The washings were added to the soap solution, the fatty acids liberated, extracted with ether, dried over anhydrous sodium sulphate and finally the ether evaporated and the acids weighed. The ether was evaporated from the solution of unsaponifiable matter, the residue taken up with pure dry ether and both acids and unsaponifiable matter were dried to constant weight at the laboratory temperature in a vacuum desiccator. The experiments were carried out at the temperature of the laboratory but in the cold weather they were left near the radiator where the temperature recorded was about 25°.

The Carbohydrate Content of Yeast.

In our earlier experiments the yeast obtained from the brewery was washed several times, filtered and used directly for the experiment; the carbohydrate of such a yeast was usually from 25 to 40 % of the dry weight. In such a case there is nearly always a fall in the carbohydrate after the yeast has been incubated in the acetate or other solution, but the loss of stored carbohydrate in the yeast which has been incubated in a solution of acetate is much less than in a yeast which has been incubated in water. When yeast is incubated in water to which oxygen is freely supplied carbohydrate is being continually decomposed during the period of incubation and carbon dioxide given off. Although at the end of the incubation period in the medium less carbohydrate may be present than in the original yeast, carbohydrate may have been formed and again decomposed: it is better therefore to start with a yeast with a low carbohydrate content, because on subsequently incubating this in the solution to be tested any increase of stored carbohydrate is more readily detected. Such a yeast may be obtained by incubating it in water for some hours, generally overnight, and passing a fairly rapid current of oxygen through the water. Part of the carbohydrate is thus oxidised and part fermented and a yeast with a low carbohydrate content is obtained. It is clear, however, that the final amount of carbohydrate represents a balance and it will be less than the sum of the initial amount of carbohydrate together with any new carbohydrate formed during the experiment by the amount of carbohydrate oxidised or fermented during the course of the experiment. It is probable that some of the substances tested may have been directly oxidised by the yeast and have thus acted as carbohydrate sparers but there is no evidence which will enable us to decide whether this is the case or whether the substance was first converted into carbohydrate and then burnt as such.

Compounds containing Two Carbon Atoms.

The two-carbon atom compounds investigated were ethyl alcohol, acetaldehyde, glycol and the sodium salts of acetic, glycollic, glyoxylic and oxalic acids. The only two of these which were readily used by the yeast and which led to the storage of notable quantities of both fat and carbohydrate were ethyl alcohol and sodium acetate. The amounts stored depend largely on the rate of the oxygen supply: at first we found that the acetate produced more storage of carbohydrate than the alcohol, but when the oxygen supply was increased in both cases the difference was removed; the oxidation is aided by shaking, but if the oxygen is bubbled through at the rate of about 6 litres per hour the supply is sufficient and the results obtained are in close agreement whether alcohol or sodium acetate be contained in the medium.

Acetaldehyde tends to act injuriously on the yeast cells, for the effects produced by incubating yeast in a solution of acetaldehyde were never very successful. The yeast only seems able to tolerate the aldehyde at very low

concentrations: it is probable that conditions of administering the aldehyde can be found which may give better results but, although at low concentrations of aldehyde some storage of fat took place, the results were never so favourable as when the medium contained acetic acid or alcohol. Lieben [1923] also found that acetaldehyde was very little used by the yeast cells, most of it remaining in the yeast filtrate.

Table I. *The effect of incubating yeast in solutions of two-carbon compounds.*

12·5 g. yeast (after incubating in oxygenated water) contain			12·5 g. yeast incubated in 1500 cc. of a solution of			Change in		
Carbohydrate g.	Fat g.	Protein g.	%			Carbohydrate g.	Fat g.	Protein g.
Oxygen rate slow.								
0·76	0·13	1·46	0·15	Ethyl alcohol		−0·10	+0·11	−0·16
0·69	0·12	1·28	0·15	,,	,,	−0·19	+0·14	−0·02
0·53	0·16	1·42	0·17	,,	,,	−0·13	+0·14	−0·10
0·69	0·12	1·28	0·30	,,	,,	−0·29	+0·14	−0·12
0·48	0·15	1·31	0·17	,,	,,	+0·04	+0·12	−0·20
0·43	0·07	1·33	0·17	,,	,,	+0·02	+0·24	−0·06
Oxygen rate 13 cc. per min.								
0·49	0·13	1·57	0·17	,,	,,	−0·19	+0·08	−0·20
Oxygen rate 70 cc. per min.						−0·11	+0·17	−0·14
Oxygen rate about 100 cc. per min.								
0·75	0·12	1·71	0·17	,,	,,	−0·15	+0·13	−0·13
0·52	0·12	1·79	0·25	,,	,,	+0·04	+0·34	−0·18
0·30	0·14	1·71	0·25	,,	,,	+0·19	+0·26	−0·22
0·43	0·11	1·47	0·30	,,	,,	+0·12	+0·27	−0·00
0·46	0·09	1·60	0·30	,,	,,	+0·18	+0·36	−0·07
Oxygen rate slow.								
0·76	0·13	1·46	0·20	Sodium acetate		−0·12	+0·14	−0·16
0·53	0·16	1·42	—			−0·03	+0·06	−0·16
0·48	0·15	1·31	—			−0·04	+0·12	−0·20
0·46	0·14	1·39	0·20	,,	,,	+0·14	+0·13	−0·02
,,	,,	,,	0·60	,,	,,	+0·07	+0·16	−0·04
,,	,,	,,	0·80	,,	,,	+0·03	+0·17	−0·02
0·40	0·11	1·31	0·20	,,	,,	+0·07	+0·20	−0·11
Oxygen rate about 10 cc. per min.								
0·30	0·10	1·71	0·17	,,	,,	−0·04	+0·04	−0·05
Oxygen rate about 100 cc. per min.						+0·20	+0·14	−0·04
0·52	0·12	1·79	0·21	,,	,,	+0·03	+0·24	−0·15
0·30	0·14	1·71	0·21	,,	,,	+0·17	+0·13	−0·26
0·76	0·10	1·60	0·21	,,	,,	−0·17	+0·29	−0·12
0·44	0·08	1·63	0·21	,,	,,	+0·00	+0·22	−0·18
0·70	0·12	1·56	0·21	,,	,,	−0·02	+0·27	−0·03
0·43	0·07	1·33	0·17	Ethyl alcohol		+0·02	+0·28	−0·06
,,	,,	,,	0·20	Glycol		−0·09	+0·05	−0·26
,,	,,	,,	0·10	Glycollic aldehyde		−0·24	+0·03	−0·07
,,	,,	,,	0·25	Na glycollate		−0·17	+0·05	−0·12
1·36	0·13	1·20	0·20	Na acetate		+0·07	+0·19	−0·11
,,	,,	,,	0·25	Na glycollate		−0·17	+0·01	−0·12
,,	,,	,,	0·20	Na glyoxylate		−0·16	+0·01	−0·12
1·36	0·13	1·20	0·15*	Acetaldehyde		−0·81	+0·06	−0·04
,,	,,	,,	0·27	,,		−0·80	+0·02	−0·06
0·73	0·09	1·33	—			−0·59	+0·03	−0·03

* The initial concentration of aldehyde was 0·06 %; acetaldehyde was then bubbled in during the course of the experiment.

Compounds containing Three Carbon Atoms.

The three-carbon atom compounds chosen for examination were acetone, glycerol and the sodium salts of lactic and pyruvic acids. Earlier experiments [Smedley MacLean and Hoffert, 1923] had indicated that fat could be formed from lactate and pyruvate and the effects of these two substances were now compared with those of alcohol and acetate. Fürth and Lieben [1922] have shown that lactate is readily used by yeast and have deduced the formation of a non-hydrolysable carbohydrate in yeast after its incubation in the oxygenated lactate solution. We found that the lactic acid is readily used by the yeast, carbon dioxide is given off and both acetic and pyruvic acids can be detected in the medium. Kayser [1924] also detected both these acids in a solution of calcium lactate which had been acted on by yeast. Whereas shaking the solution of acetate in which yeast is incubated produces comparatively little effect on the amounts of carbohydrate and fat stored by the yeast, provided that a good current of oxygen is maintained, shaking a corresponding solution of lactate during the period of incubation leads to a much increased storage of both fat and carbohydrate, and the lactate then appears to be as effective as the acetate. When the effects of the yeast on equal quantities of lactate and acetate are compared, more of the acetate disappears than of the lactate and it seems possible that the lactate is converted into acetate before it is finally burnt to carbon dioxide. We were able to make out a satisfactory carbon balance sheet in which the carbon of all the lactate which had disappeared could be satisfactorily accounted for as CO_2, as stored fat and hydrolysable carbohydrate and as small quantities of volatile acids.

Lieben [1923] also examined the action of yeast on an oxygenated solution of sodium pyruvate and found that solutions of lactate and pyruvate behaved similarly. We found that, if a moderate current of oxygen is passed through the solution and similar amounts of yeast are incubated in solutions of acetate and pyruvate respectively, the acetate is much more efficient in producing storage compounds in the yeast. Pyruvate is also less efficient in this respect than a corresponding solution of lactate. In experiments, however, in which the solution was vigorously shaken during the course of the experiment, the effect on the fat formation was very similar to that of the acetate. Probably the acetaldehyde which Neuberg and Hildesheimer [1911] have shown to be formed from pyruvic acid by the action of carboxylase acts injuriously on the yeast cells if it is allowed to accumulate. With glycerol and acetone negative results were obtained and there was no evidence that either of these could be utilised to any appreciable extent by the yeast.

Compounds containing Four Carbon Atoms.

The four-carbon atom compounds examined include aldol and the sodium salts of hydroxybutyric, acetoacetic and butyric acids. With all these, negative results were obtained (Tables IV and V) and there was no evidence of any

Table II. *Effects of acetate, lactate and pyruvate compared.*

Original yeast first incubated in oxygenated water			Change in 12·5 g. yeast after being incubated in 1500 cc. of a solution of					
			(*a*) Na acetate (0·20 % acetic)			(*b*) Na lactate (0·20 % lactic)		
Carbohydrate g.	Fat g.	Protein g.	Carbohydrate g.	Fat g.	Protein g.	Carbohydrate g.	Fat g.	Protein g.
0·53	0·16	1·42	−0·03	+0·06	−0·16	−0·07	+0·07	−0·09
0·48	0·15	1·31	+0·04	+0·12	−0·20	−0·01	+0·05	−0·09
0·70	0·12	1·56	−0·02	+0·27	−0·03	−0·04	+0·09	−0·07
0·43	0·15	1·58	−0·07	+0·13	−0·14	−0·07	+0·04	−0·11
0·27	0·15	1·50	+0·31	+0·21	−0·10	+0·18	+0·03	−0·06
x	0·08	1·43	+0·32 − *x*	+0·22	±0·00	+0·46 − *x*	+0·13	±0·00
			2·11 g. acetate in 250 cc. water, shaken for 46 hours			3·85 g. lactate in 250 cc. water, shaken for 46 hours		
*0·70	0·08	1·30	−0·48	+0·15	−0·03	−0·50	+0·17	−0·03
*0·78	0·07	1·29	−0·45	+0·16	−0·01	−0·52	+0·18	−0·01
			(*a*) Na acetate			(*c*) Na pyruvate		
*0·98	0·07	—	−0·71	+0·15	—	−0·76	+0·10	—
0·53	0·17	1·43	+0·18	+0·18	−0·01	−0·11	+0·01	−0·11
			(*b*) Na lactate					
0·49	0·13	1·50	+0·02	+0·05	−0·04	−0·13	+0·01	−0·07
			2·3 g. Na acetate in 250 cc. solution, shaken for 46 hours			2·3 g. Na pyruvate in 250 cc. solution, shaken for 46 hours		
*1·02	0·07	1·24	−0·82	+0·21	−0·06	−0·23	+0·21	—

* Yeast not previously incubated in oxygenated water.

Table III. *Carbon balance of yeast after incubation in solutions of sodium acetate, lactate and pyruvate respectively.*

	Original yeast		Yeast after incubation	
(*a*) Sodium lactate.				
Carbohydrate	0·78 g.	= 0·312 g. C	0·22 g.	= 0·088 g. C
Fat	0·07 „	= 0·052 „	0·25 „	= 0·188 „
Protein	1·29 „	= 0·645 „	1·13 „	= 0·650 „
CO_2 (free)	0·02 „	= 0·006 „	1·03 „	= 0·281 „
„ (bound)	—	—	0·32 „	= 0·087 „
Volatile acids (acetic and pyruvic)	—	—	0·06 „	= 0·024 „
Total lactic acid in original medium	2·71 „	= 1·084 „	—	—
Residual lactic acid	—	—	1·78 „	= 0·712 „
		2·099 „		2·030 „
(*b*) Sodium acetate.				
Carbohydrate	0·53 „	= 0·21 „	0·70 „	= 0·28 „
Fat	0·17 „	= 0·13 „	0·35 „	= 0·26 „
Protein	1·43 „	= 0·71 „	1·42 „	= 0·71 „
CO_2 (free)	0·27 „	= 0·07 „	0·70	„ = 0·69 „
„ (bound)	0·00 „	—	1·83	
Acetic acid: original	2·93 „	= 1·17 „	—	—
„ residual	—	—	0·76 „	= 0·30 „
		2·29 „		2·24 „
(*c*) Sodium pyruvate.				
Carbohydrate	0·53 „	= 0·21 „	0·42 „	= 0·17 „
Fat	0·17 „	= 0·13 „	0·18 „	= 0·11 „
Protein	1·43 „	= 0·71 „	1·32 „	= 0·66 „
CO_2 (free)	0·27 „	= 0·07 „	0·61	„ = 0·38 „
„ (bound)	0·00	—	0·79	
Pyruvic acid: original	3·30 „	= 1·35 „	—	—
„ residual	—	—	2·63 „	= 1·08 „
		2·47 „		2·40 „

increased storage of either fat or carbohydrate by the yeast which could be regarded as significant.

The results in the literature as to the effect of yeast on a solution of acetoacetic acid are conflicting; our results support the view that acetoacetic acid is not utilised by yeast to form storage products. Lundin [1923] found no increase in the amount of free acetone when a solution of potassium acetoacetate was acted upon by yeast and concluded that either acetone is not formed or if formed is either assimilated or burnt by the yeast cells. He found that in an oxygenated solution of acetoacetic acid (0·95 %) about 17 %, in the non-oxygenated solution about 11 %, of the acetoacetic acid had disappeared: in another experiment where the solution contained 2·93 % of acetoacetic acid the respective quantities which disappeared from the oxygenated and non-oxygenated solutions were 7·8 and 6·2 %. Lundin regarded the acetoacetic acid as being utilised by the yeast to form carbohydrate; he found an increase of dry weight had taken place in the yeast after incubation in the oxygenated acetoacetate solution and considered that this increase was due to the formation of carbohydrate, an assumption which was not confirmed by direct experiment. In any case only a small proportion of the acetoacetic acid present is acted on by the yeast.

In our experiments, after incubating the yeast for 22 hours in a solution of acetoacetate, a loss of only about 6 % of the acid originally present was observed and there was no indication that the yeast incubated in the acetoacetate solution had increased its store of carbohydrate or fat when compared with the control: our results indicate that acetoacetic acid is only utilised by yeast to a very small extent; we could not find any evidence that incubation in the sodium acetoacetate solution led to the formation of storage products in the yeast cells.

Table IV. *Comparison of original acetoacetate solution with solution after the yeast has been incubated in it.*

	(*a*) Original	(*b*) After incubation
Free CO_2	0·00 g.	0·80
Bound CO_2	0·70	0·56 g.
Total CO_2	0·70 g.	1·36 g.
Free acetone in medium and bubbler	0·09 g.	0·60
Acetone from acetoacetic acid	1·67	1·05
Total acetone	1·76 g.	1·65 g.

Amount of acetoacetic acid which has disappeared = 0·19 g.
" " " originally present = 2·94 "

	Original	After incubation in (*a*) Acetoacetate	After incubation in (*b*) Control alcohol
	g.	g.	g.
Dry weight yeast	2·07	2·40	2·68
Protein	1·35	1·31	1·32
Carbohydrate	0·36	0·38	0·40
Fat	0·10	0·23	0·27

No evidence of storage of carbohydrate or fat detected.

Table V. *Incubation of yeast in solutions of butyrate, β-hydroxybutyrate and aldol.*

	Original yeast			Yeast after incubation		
	Protein g.	Carbohydrate g.	Fat g.	Protein g.	Carbohydrate g.	Fat g.
Aldol	1·65	0·32	0·09	1·46	0·19	0·09
Acetate	1·49	0·49	0·13	1·37	0·45	0·27
Butyrate	—	—	—	1·18	0·36	0·16
Hydroxybutyrate	—	—	—	1·21	0·31	0·15
Butyrate	1·50	0·23	0·11	1·33	0·19	0·12
Acetate	1·66	0·37	0·11	1·42	0·43	0·28
Hydroxybutyrate	—	—	—	1·52	0·18	0·14

Incubation of yeast in a solution of sodium acetoacetate.

The solution of acetoacetate was obtained by adding slightly more than the calculated quantity of $N/2$ sodium hydroxide to 15·285 g. of ethyl acetoacetate in 750 cc. water and allowing it to stand overnight at the laboratory temperature: the solution was then neutralised and evaporated under reduced pressure (40 mm.) for $2\frac{1}{2}$ hours. The alcohol was determined in the solution and a corresponding amount of alcohol dissolved in water and used as a control, both solutions being made up to a litre, of which 262 cc. were diluted to 1500 cc. and used for the experiment (cp. Table IV).

Effect of Sulphite on Storage of Fat and Carbohydrate by Yeast.

As the result of these experiments we came to the conclusion that two-carbon atom compounds are more readily utilised by yeast than the three- and four-carbon atom compounds we had examined. Of the two-carbon atom compounds, acetic acid and ethyl alcohol furnish equally efficient sources of building material for the formation of the fat and carbohydrate the yeast stores within its cell. It seemed to us possible that both these acted as potential sources of acetaldehyde, supplying it in such a way that it was never allowed to accumulate in the cell but was immediately condensed to some less injurious product.

Neuberg has shown in his work on fermentation that acetaldehyde is formed as a decomposition product of sugar and is then reduced to alcohol; for when sodium sulphite is added to a fermenting sugar solution, the acetaldehyde reacts with the sulphite and the aldehyde-bisulphite compound can be isolated from the medium. The yield of alcohol is therefore diminished and may fall to about one-third of that which would be produced in the absence of the sulphite. There is always considerable dissociation of the bisulphite compound so that some alcohol is always formed. The excess of hydrogen which has not been used to reduce the acetaldehyde withdrawn by the sulphite is effective in producing a much increased supply of glycerol in the medium and the method has been used for the large scale preparation of glycerol. There seemed a possibility therefore that the addition of sulphite to the medium

might be used as a means of testing whether either the alcohol or acetate went through the stage of aldehyde before being converted to storage carbohydrate or fat by the yeast. If alcohol or acetic acid were first converted into acetaldehyde a considerable proportion of this would be held by the sulphite and we might expect to find a diminished store of fat or carbohydrate in the yeast; this proved to be the case.

The amounts of stored fat and carbohydrate were much less in the yeast which had been incubated in the solutions to which sulphite had been added than in the yeast incubated in the pure solutions of alcohol or acetate.

Table VI. *Effect of sulphite on storage of fat and carbohydrate by yeast.*

Original yeast		After incubation in: (*a*) Alcohol		Alcohol and sulphite	
Carbohydrate g.	Fat g.	Carbohydrate g.	Fat g.	Carbohydrate g.	Fat g.
0·435	0·11	0·545	0·38	0·29	0·26
0·46	0·09	0·68	0·43	0·28	0·21
—	—	—	—	0·28	0·22
0·71	0·08	0·54	0·40	0·33	0·29
		(*b*) Acetate		Acetate and sulphite	
0·44	0·08	0·44	0·31	0·25	0·19
		(*c*) Alcohol and sulphite		Acetate and sulphite	
0·43	0·11	0·31	0·22	0·36	0·25
0·76	0·10	0·48	0·23	0·42	0·21

The nature of the fat or rather of the ether-soluble substance was examined and proved to be practically the same in the yeast incubated in the media with and without the addition of sulphite. It consisted of from 53 to 60 % of fatty acids, having an iodine value of from 76 to 78 and of from 32 to 37 % unsaponifiable matter, which from its very high iodine value (about 200) must have consisted chiefly of the ergosterol characteristic of yeast [Smedley MacLean and Thomas, 1920].

In all these experiments however the processes of carbohydrate and fat storage always occurred together. From the results so far obtained it was possible that acetaldehyde was first formed and then built up directly either to form carbohydrate molecules or to form fatty acid chains; but there was also the possibility that the acetaldehyde was first condensed to a hexose molecule and that the latter was either further condensed to form storage carbohydrate or that the hexose molecules were directly condensed with each other to form long carbon chains which by subsequent reduction were converted to fatty acids as originally postulated by Emil Fischer.

These yeast experiments seemed for the first time to offer an opportunity of testing whether a fatty aldehyde occurred as an intermediate substance on the path from sugar to fatty acid. If this were the case the addition of sulphite to a solution of sugar in which yeast was incubated should produce a diminution of the fat and carbohydrate in yeast when compared with the same

sample of yeast incubated in a sugar solution to which sulphite had not been added. The results should be similar to those obtained when yeast is incubated in solutions of alcohol and acetate with and without the addition of sulphite which have already been described above.

It was found, however, that the addition of sulphite to a solution of fructose or glucose produces a result quite different from that obtained when the sulphite is added to the solution of alcohol or acetate. In the case of the yeast incubated in the sugar solution to which sulphite has been added there is no constant diminution in the amounts of stored fat and carbohydrate when compared with the yeast incubated in the sugar solution itself; the amount of carbohydrate stored is always greater in the yeast taken from the sulphite-containing medium, whilst the amount of fat is sometimes greater and sometimes less than the amount stored in the yeast incubated in the sugar solution. When, instead of considering the total amount of ether-soluble substance which we have termed the fat, the amount of fatty acids actually present is considered, we find the total amount of fatty acid present after the addition of the sulphite to the medium is in very much closer agreement with the corresponding amount produced in the hexose solution without the addition of sulphite. Further the effect of increasing the quantity of sulphite in the medium was to increase both the amounts of fat and of carbohydrate stored in the yeast. The fat, or rather the ether-soluble substance, isolated from the yeast after its incubation in the hexose solution differs in composition from that isolated from yeast incubated in the hexose-sulphite medium. In the latter case the percentage of fatty acids in the fat is considerably higher than in the former case and the percentage of unsaponifiable matter or sterol is considerably lower.

When yeast was incubated in a solution of fructose also containing about 4 % of a mixture of alkali phosphates, the addition of sulphite diminished the total amount of ether-soluble substance considerably, but here again the most marked diminution was in the weight of sterol, which was very much reduced. The effect on the carbohydrate was variable, and in the two experiments carried out there was a drop of about 20 % in the amount of fatty acid formed.

From these experiments it seems justifiable to infer that since the quantity of fatty acids formed from hexose by yeast is very little if at all diminished by the addition of sulphite to the medium, a fatty aldehyde does not occur as an intermediate stage between the hexose molecule and the fatty acid. The view of Fischer that the hexose chains are directly condensed to form chains containing fatty acid chains built up in multiples of six carbon atoms cannot then be disregarded. It would appear that in the action of yeast on ethyl alcohol or on acetic acid the first stage is the conversion to acetaldehyde; the next, the condensation of aldehyde to form a hexose molecule. This change does not take place at all unless there is a free supply of oxygen and oxidation must indeed form an essential part of the process for the change is represented by the following equation: $3CH_3.CHO + 3O = C_6H_{12}O_6$.

In a non-oxygenated solution of either alcohol or acetate there is no evidence of increased carbohydrate or fat storage; these materials are only formed in the presence of a plentiful oxygen supply. With an inadequate oxygen supply less carbohydrate is stored in the yeast incubated in the solution of alcohol than in the acetate solution, a result explained by the fact that oxidation of the alcohol to aldehyde has also to be brought about. We have previously shown that when yeast is incubated in a solution of a fermentable sugar the amounts of fat and carbohydrate stored are increased by the oxygenation of the medium but an appreciable amount of storage takes place in the absence of a free supply of oxygen. It seems therefore that hexose can only be formed from acetaldehyde in the presence of a free supply of oxygen, but that fat and carbohydrate can be formed from hexose in the absence of a supply of oxygen though the process is very much aided by such a supply. A plentiful supply of oxygen is an essential condition in order that good results may be obtained and in carrying out these experiments care must be taken that the oxygen is being passed through the control and the experiment at approximately the same rate.

The fact thus established that the yeast uses acetaldehyde as a starting material to build up carbohydrate is of particular interest in connection with the recent work of Neuberg and Gottschalk [1924, 1925], who have brought forward convincing evidence that acetaldehyde is a normal step in the oxidation of carbohydrate by muscle.

After these experiments were completed we noticed a paper by Haehn and Kinttof [1925] on the formation of fat by *Endomyces vernalis*. These authors find that this organism can utilise acetaldehyde, alcohol, aldol and pyruvic acid and store up fat from such solutions, carbon dioxide being simultaneously formed. They conclude that sugar is first decomposed with the production of acetaldehyde and that the latter is then condensed to produce fatty acids. Glycerol is also used by this fungus and built into fat, but less readily than alcohol. On the whole their conclusion agrees with the one that we have drawn from our experiments with yeast, namely that the simpler carbon compounds pass through the stage of acetaldehyde on their way to conversion to fat. Haehn and Kinttof also tested the effect of sulphite on the growth of *Endomyces vernalis* in a cane sugar solution; they found, however, that sulphite is very unfavourable to the growth of this organism and cannot be used in concentrations of more than 1 %. The fact that yeast is much less adversely affected by the presence of sulphite in the medium makes it easier to carry out such experiments and we think that Haehn and Kinttof's experiments with the *Endomyces* cannot be taken as furnishing conclusive evidence that the acetaldehyde passes directly to fat without being first converted to hexose. It is of course possible though unlikely that the path of metabolism in these two organisms is different. The evidence we have obtained from the study of the effect of the addition of sulphite to solutions of alcohol and sugar respectively in which yeast is incubated seems to us to point quite definitely to the con-

clusion that the hexose is transformed directly to fat without being first broken down to acetaldehyde. We feel that it is more difficult to interpret the experiments on *Endomyces* in which the sulphite is added to the sugar solution, because the conditions of growth are so adversely affected that it is necessary to prolong the experiments for very long periods in order to show the increase of fat.

We have previously brought forward evidence that phosphates [Smedley MacLean and Hoffert, 1923] play an important part in the change from hexose to fat, and that the amount of phosphate taken up by the yeast incubated in a solution containing both hexose and phosphate depends on the concentration of the *hexose*. It seems probable therefore that the hexose is converted into a hexosephosphate and that this substance undergoes further changes which result in the direct formation of the fatty acids; there is no evidence available to support the view that this compound breaks down into a simple substance from which the fatty acids are synthesised.

Table VII. *Effect of sulphite and phosphates on storage of carbohydrate, fat and sterol by yeast.*

12·5 g. pressed yeast after incubation in 1500 cc. of a solution of	Weight sulphite added ($Na_2SO_3.7H_2O$) g.	Amount carbohydrate g.	Amount ether-soluble substance g.	Saponification value	Amount fatty acids g.	% fatty acids	I.V. fatty acids	Amount sterol g.	% sterol	I.V. sterol
1. Glucose, 0·5 %	—	0·71	0·39	201·6	0·26	66·2	79·48	0·056	17·4	113·0
1 *a*. „ „	20	0·85	0·43	204·7	0·35	81·7	72·70	0·055	12·8	114·0
1 *b*. „ „	40	0·89	0·35	—	—	—	—	—	—	—
2. „ „	—	0·60	0·22*	—	—	—	—	—	—	—
2 *a*. „ „	20	0·88	0·33	220·0	0·27	81·4	72·7	0·41	12·4	—
3. Fructose, 0·5 %	—	0·82	>0·22	206·6	>0·17	79·7	66·4	>0·18	16·9	—
3 *a*. „ „	20	1·07	0·38	—	0·25	65·4	77·35	0·37	17·6	175·1
4. „ „	—	0·73	0·202	218·7	0·15	73·0	—	0·031	15·5	—
4 *a*. „ „	10	0·66	0·299	—	0·68	76·0	—	1·32	14·9	—
4 *b*. „ „	20	0·75	0·239	—						
4 *c*. „ „	30	1·04	0·354	196·0						
5. „ „	—	0·88	0·35	—	—	62·0	—	—	27·5	—
5 *a*. „ 0·25 %	30	1·06	0·28	201·9	0·20	—	76·47	0·06	20·8	155·8
5 *b*. „ 0·5 %	30	1·00	0·28	—	—	—	—	—	19·8	—
5 *c*. „ 0·75 %	30	1·29	0·35	—	—	—	—	—	—	—
6. „ 1 %	—	1·17	0·73	153·0	0·44	60·1	—	0·25	35·4	232·1
6 *a*. „ „	30	1·40	0·42	170·8	0·32	74·4	79·0	0·10	25·1	158·0
7. „ 2·4 %	—	0·97	0·55	153·2	0·35	63·7	72·01	0·18	33·1	158·6
7 *a*. „ „	60	1·65	0·67	181·8	0·48	72·6	76·47	0·14	21·7	159·1
9. Fructose, 2 % + phosphates†	—	1·15	1·15	136·4	0·60	57·9	73·24	0·37	32·1	176·3
9 *a*. Fructose, 2 % + phosphates	30	1·29	0·58	169·8	0·45	72·5	78·52	0·10	17·0	147·7
10. Fructose, 3 % + phosphates	—	1·77	0·80	149·9	0·51	65·0	76·17	0·25	31·6	205·1
10 *a*. Fructose, 3 % + phosphates	30	1·18	0·50	172·5	0·41	81·5	77·76	0·07	14·4	163·6

* Some of fat in control was lost.

† 0·39 % Na_2HPO_4 + 0·029 % KH_2PO_4.

With regard to the synthesis of sterol or unsaponifiable matter by the yeast, in every case the amount present was very much diminished in the yeast by the addition of sulphite to the hexose medium in which the yeast was incubated, a result, which suggests that the sulphite is exerting an effect on the internal metabolism of the yeast cell. There seem good grounds for believing that in the synthesis of sterol from hexose, an aldehyde forms an intermediate stage. In some cases the amount of sterol is only one-third or one-quarter that present in the yeast which has been incubated in the corresponding medium without the addition of sulphite.

Discussion of Results.

These experiments undoubtedly seem to furnish evidence that a path of chemical transformation exists from hexose to fatty acid without passing through any intermediate stage of aldehyde, and the question arises: Are we really justified in regarding the lower fatty acids containing even numbers of carbon atoms which occur in such fats as butter and coconut oil as stages in the synthesis of the fatty acids? Actually the only experimental evidence which is available is directly against this view and suggests that they are rather to be regarded as degradation products; this is furnished by some experiments of Meigs, Blatherwick and Carey [1919] on the difference of the composition of the blood before and after its passage through the active mammary glands of cows. These observers estimated the amount of inorganic phosphate and of lipin phosphate in the blood of the jugular and mammary veins of cows, both of those which were actively lactating and of those which were not giving milk at the time. They found that the inorganic phosphate in the actively lactating animal was increased and the lipin phosphorus diminished in the blood leaving the mammary gland of the actively lactating animal; they argued from this that fat is split off from the lipins of the plasma by the gland cells and they calculated that the change in lipin phosphorus would account for the setting free of the whole of the fat contained in the milk. If this conclusion be justified and if the whole of the fat be formed in this manner, the lower fatty acids present in milk must be formed by a degradation process from the higher fatty acids, for no fatty acids with less than sixteen carbon atoms have been shown to occur in lipins. Chiefly owing to the work of Levene we know that arachidonic, oleic, stearic and sometimes palmitic acids occur in the lipins of the blood, so that, if the acids containing from four to fourteen carbon atoms which are present in milk fat are derived from lipin, they must be formed by a process of degradation. One of the few facts which has been definitely established about the fate of the fatty acids is that they undergo β-oxidation in the body, and that the resulting products would therefore be the even-numbered members of the fatty acid series. Having regard to the experiments of Meigs, Blatherwick and Carey, it cannot be considered as definitely established that the lower fatty acids are intermediate

stages on their way to being built up into the higher fatty acids. The point is of fundamental importance in its bearing on the question of the method of formation of the fatty acids, but since the only two pieces of experimental evidence at present available seem to be that the higher fatty acids undergo β-oxidation in the body, presumably with formation of the lower even-numbered fatty acids as intermediate stages, and that fat is split off from the lipins of the blood by the active mammary gland, we are not justified in regarding these lower fatty acids as necessarily formed by a synthetic process.

The evidence obtainable from a study of the yeast organism is distinctly against the view that either acetaldehyde or pyruvic acid is an intermediate stage between the hexose molecule and the fatty acid. This is in agreement with the view of Shaffer [1922], who has opposed the hypothesis that fatty acids are built up from acetaldehyde on the ground that the first step would be the formation of the aldehyde of β-hydroxybutyric acid, a ketogenic substance, and this fact would render it necessary for us to regard the carbohydrates as ketogenic substances. It seems probable that the two-carbon atom compound is first converted to acetaldehyde, then synthesised to a hexose and that the hexose serves as the unit for further condensation with the ultimate production of the fatty acid. The phosphate group has previously been shown by us [1924] to play an important part in the process; it probably enters into combination with the hexose molecule or with some product derived from it, and there is no evidence that any breaking down of the six-carbon chain takes place before it is built up into the higher fatty acids.

There is certainly a good deal to be said in favour of the view that the hexose molecules are able to join directly together, in a way which we cannot imitate in the laboratory, to form long chains of carbon atoms linked together in multiples of six.

Summary.

1. The action of yeast on the following substances has been investigated: ethyl alcohol, acetaldehyde, sodium acetate, glycol, glycollic aldehyde, sodium glycollate, sodium glyoxylate, sodium oxalate, sodium lactate, sodium pyruvate, acetone, glycerol, aldol, sodium butyrate, sodium β-hydroxybutyrate, sodium acetoacetate.

2. Of these ethyl alcohol and sodium acetate were found to be best utilised by the yeast to form storage fat and carbohydrate, part being at the same time burnt and carbon dioxide formed.

3. Sodium lactate is also used by the yeast to build up fat and carbohydrate and to form carbon dioxide; the action is increased by vigorously shaking the solution and then resembles that of the acetate. Sodium pyruvate can also be utilised; the action is increased by vigorously shaking the solution but it is not as favourable as the lactate. Probably the acetaldehyde known to be formed acts unfavourably on the yeast, as we find that even at low con-

centrations the presence of acetaldehyde in the solution is unfavourable to the formation of metabolic products by yeast.

4. In all cases there must be a plentiful supply of oxygen to the medium, without which no storage takes place in the yeast.

5. The addition of sodium sulphite to the alcohol or acetate medium diminishes the storage of both fat and carbohydrate and it is inferred that the first stage is the transformation of the alcohol and acetate to acetaldehyde, which however is never allowed to reach a concentration which becomes injurious to the action of the cell. The ether-soluble substance formed in this way contains the same proportion of unsaponifiable matter (sterol) and fatty acids as that formed from the alcohol or acetate in the absence of sulphite.

6. The addition of sulphite to a solution of hexose in which yeast is incubated results in an *increase* of stored carbohydrate; the ether-soluble substance may be increased or diminished; the proportion of fatty acids it contains is considerably raised and the proportion of sterol much diminished. On the whole the amount of fatty acids formed is little influenced by the addition of sulphite to a medium containing hexose.

7. The deduction is made that the acetaldehyde is first condensed to hexose and that the latter is either converted to storage carbohydrate or else directly condensed to form the higher fatty acids without passing through a fatty aldehyde stage, probably by a direct linking of the hexose molecules.

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XLVII. THE ACTION OF YEAST ON LACTIC ACID.

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The fact that lactic acid occurs in small quantities in the fermentation liquors of yeast has caused it to be regarded by some investigators as an intermediate stage in the breakdown of carbohydrates during fermentation and various observers have shown that yeast can utilise lactic acid when it is directly supplied with the lactic acid in a suitable form. Slator found that yeast had little action on the free acid [1907], but three years later Buchner and Meisenheimer [1910] showed that whilst the free acid was not attacked by yeast the sodium salt was destroyed.

Fürth and Lieben have studied the question in considerable detail [1922, 1]. They found that when a plentiful supply of oxygen was given, lactic acid in the form of its sodium or lithium salt was readily destroyed by yeast. It was necessary to have a vigorous current of oxygen passing into a continually shaken yeast suspension. Under these conditions lactic acid disappeared from the medium with simultaneous development of CO_2. Fürth and Lieben recognised that some of this carbon dioxide came from the carbohydrate of the yeast. They estimated this carbohydrate before and after the yeast had been incubated in the lactate solution by hydrolysing the yeast with 2·2 % HCl for 4 hours on a water-bath. There was always a decrease of carbohydrate during the experiment. None of the lactic acid which disappeared from the solution could therefore be accounted for by formation of new hydrolysable carbohydrate in the yeast.

The destruction of lactic acid by yeast has been confirmed by Myrbäck and Everitt [1924], by Kayser [1924] and by Smedley MacLean and Hoffert [1923]. Table I contains the results of typical experiments carried out with sodium lactate and with the free acid.

Without oxygen very little change occurred in the yeast or in the lactate medium; free lactic acid was only very slightly attacked either with or without oxygen.

Fürth and Lieben were unable to account for all the lactic acid which disappeared from a solution of sodium lactate when it was acted upon by yeast: it was not built up into an easily hydrolysable carbohydrate in the yeast nor was it completely burnt to carbon dioxide, for the amount of carbon

[1] This paper contains some of the work submitted for the degree of Ph.D. of the University of London.

Table I.

(Figures are g. per 12·5 g. of yeast.)

Original yeast				Yeast after incubation in sodium lactate with current of oxygen				Yeast after incubation in sodium lactate with current of hydrogen			
Carbohydrate	Fat	Protein	Lactic acid given per 250 cc.	Carbohydrate	Fat	Protein	Loss of acid	Carbohydrate	Fat	Protein	Loss of acid
0·82	0·092	1·41	1·24	0·21	0·242	1·47	1·15	0·52	0·092	1·46	0·13
				Yeast after incubation in 1 % lactic acid with current of oxygen				Yeast after incubation in 1 % lactic acid with current of hydrogen			
0·98	0·065	1·34	2·85	0·40	0·072	1·09	0·41	0·60	0·074	1·07	0·20

dioxide was never sufficient to account for all the lactic acid which had disappeared. They therefore sought to obtain further information about the fate of the lactic acid and published the result of their investigations in a second paper [1922, 2]. The method they employed was to make a carbon balance, putting on one side of the balance sheet the carbon of the lactic acid which had disappeared and on the other the carbon in the compounds formed in the yeast suspension after incubation. They ran two experiments under identical conditions, *A*, in which yeast was incubated in sodium lactate solution and, *B*, in which the yeast was incubated in water only. They assumed that the changes which took place in the yeast in *A* were the same as those that occurred in the yeast in *B*, so that the difference between the amounts of carbon dioxide obtained in the two experiments could be attributed to the lactic acid which disappeared from *A*. In this experiment *A*, besides the carbon dioxide which was given off, some carbon dioxide was retained in the solution as bicarbonate, being bound by the soda which was set free when the lactic acid was destroyed. Fürth and Lieben generally calculated this amount of carbon dioxide from the loss of lactic acid which they found experimentally. When they made their balance based in this way on differences between the two experiments *A* and *B* with the lactic acid lost on the one side and the CO_2 found on the other, there was always a large deficit of carbon which could not be accounted for. From one or two experiments which these investigators carried out, they considered that no fat was formed in the yeast and that volatile substances, such as aldehyde, alcohol or acetic acid, occurred in such small amounts that they were negligible in comparison with the deficit.

Fürth and Lieben therefore put forward the view that a non-hydrolysable carbohydrate must be formed in the yeast from the lactic acid or else an increase in the carbon content of the yeast protein must have taken place. The dry weights of the yeasts were determined; that of the yeast in experiment *A* was larger than that in *B* and if this difference were calculated as cellulose the deficit disappeared. These experiments were regarded by the authors as providing sufficient evidence to justify their conclusions. In the experiments carried out by Smedley MacLean and Hoffert [1923], whenever

yeast was incubated in an oxygenated solution of sodium lactate, a certain amount of fat was always formed and could not be ignored in making the carbon balance sheet. Some of Fürth and Lieben's experiments were therefore repeated, certain modifications, which did not affect the conditions of the experiments but only the methods of estimating the final products, being introduced. In a typical experiment a quantity of 25 g. of yeast was first incubated for 7 hours in tap water, with vigorous shaking in oxygen. It was then left overnight and shaken the next day for 2 hours. 3·32 g. of sodium lactate solution containing 1·83 g. of lactic acid were added and the yeast again shaken with oxygen for a further 7 hours. The experiment was again left for a night and worked up on the following day. During the whole time the CO_2 given off was collected in receivers containing baryta about $N/4$ in strength, a sufficient number being connected so that the last two remained clear. A parallel experiment in which the yeast was incubated in water without the lactate was carried out at the same time.

After incubation the yeast was filtered off, well washed with CO_2-free water, and the liquid from it made up to a known volume with CO_2-free water. Aliquot parts were used for determining residual lactic acid, volatile acids, alcohol, non-volatile acids and CO_2 present in combination as bicarbonate or carbonate. The yeast was analysed by the method used by Smedley MacLean and Hoffert [1923], and hydrolysable carbohydrate, fat and protein determined both before and after incubation.

In the majority of experiments the bound CO_2 in the liquid was present as bicarbonate. It was determined (1) directly by acidifying with phosphoric acid, removing it by CO_2-free air or oxygen and collecting it in standard baryta; (2) by determining its equivalent amount of alkali; the liquid was boiled under a reflux condenser with excess of standard acid until the CO_2 was removed; the alkali thus set free neutralised an amount of acid which could be found by back titration with standard alkali.

The lactic acid was estimated by Long's modification [1924] of Clausen's oxidation method, after clearing the liquid with sodium tungstate and copper sulphate and lime. Preliminary experiments with pure zinc lactate gave an 87 % yield of the theoretical amount of lactic acid. Therefore, 100/87 was taken as the factor in reckoning the lactic acid.

The volatile acids were measured by distillation with phosphoric acid, and titration of the distillate with standard alkali. Lactic acid was determined in the distillate. From the weight of the sodium salts obtained on evaporating the neutralised distillate to dryness, the volatile acid, other than a small quantity of lactic acid carried over, was found to be acetic acid.

Pyruvic acid was determined qualitatively by testing with sodium nitroprusside and ammonia, and quantitatively by Smedley and Lubrzynska's method [1913].

Alcohol was estimated by Hamill's modification [1909] of the Nicloux method. The amount was generally so small as to be negligible.

In the experiment with sodium lactate in which lactic acid was destroyed each molecule of acid disappearing freed an atom of sodium to hold CO_2 in solution as bicarbonate or carbonate and it was therefore possible to calculate from the bound CO_2 the loss of lactic acid which had occurred. It was always found to be less than that given by actual determination of the acid by Clausen's method. The difference was accounted for by the production of other acids, *e.g.* acetic acid, pyruvic acid and traces of succinic acid, which were present in such quantities that they could not be neglected. Kayser [1924], in his investigation of the action of yeast on calcium lactate solution, found appreciable amounts of pyruvic, acetic and valeric acids in the medium.

A wet combustion of the residue from the evaporated liquids gave a small amount of carbon not accounted for by any of the above-mentioned substances. The amount was the same in the experiment with water as in that with sodium lactate.

Table II.

25 g. original yeast			25 g. yeast incubated in sodium lactate solution *A*			25 g. yeast incubated in water *B*		
	g.	Carbon g.		g.	Carbon g.		g.	Carbon g.
Carbohydrate	1·48	0·59	Carbohydrate	0·48	0·19	Carbohydrate	0·42	0·16
Fat	0·171	0·13	Fat	0·29	0·22	Fat	0·23	0·17
Protein	2·46	1·23	Protein	2·34	1·17	Protein	2·27	1·13
CO_2 (free) in tap water	0·029	0·01	CO_2 during 1st period in H_2O	0·850	—	CO_2 during 1st period	1·049	—
„ (bound) in tap water			CO_2 during 2nd period with lactate	0·790	—	CO_2 during 2nd period	0·373	—
			Bound CO_2 in liquid	0·424	—	CO_2 in liquid	0·08	—
			Total	2·064	0·56	Total	1·502	0·41
Lactic acid	1·83	0·73	Acetic acid	0·14	0·06	Acetic acid	0·02	0·01
			Pyruvic acid	0·12	0·05	Pyruvic acid	0·02	0·01
			Residue of carbon in liquid	—	0·06	Residue of carbon in liquid	—	0·06
			Residual lactic acid	0·83	0·33			
Total carbon for lactate, Exp. *A*	—	2·69	Total carbon		2·64			1·95
Total carbon for water, Exp. *B*	—	1·96						

In Table II are the figures of a typical experiment carried out after numerous preliminary experiments had established the technique of the methods. In making the balance, the carbon of the carbohydrate, fat and protein present in the yeast at the beginning of the experiment and of the lactic acid used during incubation is put down on one side. On the other side are the carbon of the carbohydrate, fat and protein of the yeast after incubation, the carbon of the carbon dioxide and of all the substances which were found in the medium. It will be seen that all the carbon of the lactic acid which has been destroyed can be accounted for within the limits of experimental error. Under the conditions of Fürth and Lieben's experiments, *i.e.* the incubation of the yeast in a small bulk of somewhat concentrated solution, the amount of fat

formed is very small, much less than that produced under the conditions of the experiments carried out by Smedley MacLean and Hoffert. For the favourable development of fat, incubation of the yeast in a large volume of a dilute solution of lactate appears to be necessary. There was no evidence that any of the carbon of the lactic acid had gone to form a non-hydrolysable carbohydrate or new protein.

Table III. *Balance according to Fürth and Lieben's method of calculation.*

	g.	Carbon g.		g.	Carbon g.
Total lactic acid	1·83	0·73	Residual lactic acid	0·83	0·33
			Extra CO_2 compared with control	0·22	0·06
			Maximum retention of CO_2	0·49	0·13
		0·73			0·52
				Deficit	0·21

For comparison with Table II a balance sheet has been drawn up according to the method used by Fürth and Lieben and is given in Table III. The figure for CO_2 is obtained from the difference in free CO_2 between experiment *A* in which the yeast was incubated in lactate and *B* in which the yeast was incubated in water. The maximum retention of CO_2 is calculated, as described above, from the lactic acid which disappeared. When the balance is made in this way a deficit of 0·21 g. carbon is obtained. It seems, therefore, that the method of balancing by differences may introduce considerable error. This is supported by the fact that in one of Fürth and Lieben's experiments in which they did not balance on the differences between the control and the experiment but used the method of calculation shown in Table II they did not get their usual large deficit of carbon.

The experiment quoted above and the two balance sheets show clearly that, whilst lactic acid is undoubtedly destroyed by yeast, the evidence available does not make it necessary to assume either the formation of a non-hydrolysable carbohydrate or the formation of protein with a higher carbohydrate content.

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XLVIII. THE EFFECT OF ULTRA-VIOLET LIGHT ON THE CALCIUM AND PHOSPHORUS METABOLISM OF THE LACTATING ANIMAL.

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In growth, in pregnancy and in lactation the animal requirements for calcium and phosphorus are relatively great. This is particularly the case in farm animals in many of which powers of rapid growth, frequent pregnancies and heavy lactations are the characteristics sought after. Apart from the composition of the diet, ultra-violet light would seem to be the factor which most profoundly affects the metabolism of calcium and phosphorus. In 1924 we [Orr, Magee and Henderson, 1924] showed that in the case of the growing pig irradiation by a carbon arc lamp increased the absorption of calcium and phosphorus as estimated by balance experiments and subsequent analysis of the bones. The work of Maynard, Goldberg and Miller [1925] on the prevention of "stiffness" in swine by exposure to sunlight largely confirms our results.

It is generally believed that in a milking animal there is a storing up of calcium and phosphorus during the pre-lactation stage. In heavy milkers they are secreted in such amount that during early lactation an animal fed on an ordinary practical ration is commonly on a negative balance, particularly with regard to calcium. The difficulty of reducing this negative balance has been frequently noted. Thus Forbes *et al.* [1922] found that, despite superabundant supplies of calcium, phosphorus and other minerals, the calcium balances remained negative in the first half of lactation. Hart, too, and his associates [1922, 2] showed that, whilst substituting alfalfa hay for timothy hay in the ration reduced the negative calcium balances of milking cows, it did not render them positive. A supplement of bone meal added to timothy hay had the same effect. On the other hand, Hart and his co-workers [1922, 1] had previously succeeded in obtaining positive calcium balances by feeding alfalfa hay. The non-success of the later experiments they attribute to the method of curing the hay and they believe that there had been a "destruction during the curing process of the vitamin assisting calcium assimilation." This would help to explain the earlier work of Steenbock and Hart [1913] in which the good effect of green pasture on the calcium retention of milking goats was noted. The view is further supported by an investigation by Gaessler and McCandlish [1923] in which positive calcium balances were

obtained on feeding fresh alfalfa but negative balances when old alfalfa was used. Further, Miller, Brandt and Jones [1924] have demonstrated the beneficial effect of green kale upon the mineral balances in early lactation. Munroe [1924] has obtained calcium storage in liberally milking cows, a result which he tentatively ascribes to "some organic factor assisting calcium assimilation" in the large amount of clover hay being fed. Hart, Steenbock and Hoppert [1921] have shown that in milking goats the administration of cod-liver oil (5 to 10 cc. per day) may convert negative to positive calcium balances. The oil, however, was not very well tolerated.

Arising out of metabolic experiments with lactating goats, Hunt, Winter, Schultz and Miller [1923] suggest that "the effect of green feed in causing the difference in assimilation of calcium over that of dry feed is due to the fact, other than the vitamin theory, that the calcium in the cells of green plants and hay not exposed to an excessive drying is more highly dispersed and therefore better digested and assimilated than the calcium in dry hay." They believe this theory to be strengthened by the fact that tricalcium phosphate precipitated in a starch paste and added to a grain and hay ration brought about positive balances in lactating goats [Hunt, Winter and Miller, 1923].

Reference must also be made to the work of Meigs and his associates [1919]. They are of opinion that the experimental procedure as carried out in metabolic experiments with cows is largely responsible for the negative calcium balances so frequently found.

In brief, then, it has been shown or suggested that the following factors influence the absorption of calcium and phosphorus by the lactating animal: (1) stage of lactation; (2) calcium and phosphorus content of the ration; (3) nature of the organic constituents of the diet, either as regards their content of anti-rachitic factor, or as regards the digestibility of their calcium and phosphorus; (4) a nervous factor.

In none of the above-described experiments had the effect of sunlight or its absence been directly considered. It seemed to us that this might be a factor further complicating an already complicated problem. In view of our previous findings as regards the stimulus to calcium and phosphorus retention induced by irradiation of the growing pig, several experiments on the effect of ultra-violet light on the mineral metabolism of lactating goats were carried out. Meanwhile Hart, Steenbock and Elvehjem [1924] published results which showed that ultra-violet irradiation may convert negative balances of calcium and phosphorus in the lactating goat to positive balances. In a preliminary note we [Orr, Magee and Henderson, 1925] described three of our experiments which were then completed. The present paper describes these investigations in somewhat greater detail and also a fourth experiment which was in progress when the note was published.

Experimental Work.

The technique employed throughout the four experiments was in general the same. Any minor variations are noted under the respective experiments.

Method.

Animals. Two lactating goats, of a Toggenberg strain not pure bred, were used, one a black and white animal, the other light grey in colour. Their coats were not long and as stated were comparatively light in colour but in no cases were the animals closely cropped or shaved.

Housing. Prior to each experiment the animal concerned occupied an inside pen for some time. Thereafter it was housed in a metabolism cage in the animal room. All light entering the room traversed glass. Before analyses were begun several days were allowed for the animal to become accustomed to its environment and diet.

Diet. This varied as shown under each experiment, but in the main consisted of a ration of hay and concentrates. Distilled water was given *ad lib.* Before each experiment the calcium, phosphorus and nitrogen content of each constituent of the diet was determined. In the case of the hay, the moisture content was also estimated. On the day the samples were taken for analysis the daily rations of hay for the whole duration of the experiment were weighed out and stored in separate bags; thus difficulties due to variations in moisture content from day to day were avoided. Any hay scraps left by the goat during a collection period were weighed and the percentage moisture estimated. From this the weight of calcium, phosphorus and nitrogen unconsumed was calculated and allowance made.

Collection of excreta and milk. The general routine employed in the collection of excreta was that in common use at the Rowett Institute [Husband, Godden and Richards, 1923]. Urine and faeces were measured and weighed daily and one-quarter of the urine and one-tenth of the faeces kept in a cool place, using a few drops of toluene as preservative. These aliquot portions were thoroughly mixed at the end of the 4 days, which generally constituted a collection period, and samples were taken for analysis.

The goat was milked twice daily, at 9 a.m. and 5 p.m. The same milker continued throughout an experiment. The volume at each milking was recorded and the milk with the addition of a few drops of formalin stored in a cool place till 4 days' milkings had been collected. The analyses were carried out on samples from this 4 days' total.

Analyses. Calcium was precipitated as oxalate and estimated volumetrically. For phosphorus the modified Pemberton-Neumann method was employed [Richards and Godden, 1924] and in Exp. 4 Briggs' modification of the Bell-Doisy technique was also used.

Irradiation. In all four experiments an irradiation period (varying from 14 to 21 days) was interposed between a pre- and a post-period of about equal duration.

The irradiation period consisted of 4 hours' daily exposure to the carbon arc at 4 feet distance. The current consumption at 230 volts a.c. was 10 amperes when the lamp was consuming 45[1].

Experiment 1.

The light grey goat, in the late lactation stage and yielding about 120 cc. per day, was used. The periods were as follows: (1) a preliminary period of 28 days, during which the goat was in an inside pen; (2) a pre-period of 11 days; (3) an irradiation period of 14 days and (4) a short post-period of 5 days. Collection and analyses were carried out on the 3rd and 4th days alternately, except in the post-period when they were on the 3rd and 5th days of that period.

Diet.

Food	Weight in g.	% CaO	% P_2O_5
Hay	900	1·2810	0·4019
Oats	70	0·1158	0·6679
Linseed meal	25	0·3965	0·9306
Maize meal	150	0·0110	0·5395
Total g. per day	1145	11·7257	5·1263

Results. It will be seen from Table II, Exp. 1, and also from the accompanying graph that whilst the CaO and P_2O_5 balances remained negative throughout the experiment the CaO balance was less negative during the irradiation period. The reverse is true of the P_2O_5 balance. No significant change is seen in the composition of the urine or in the volume and composition of the milk during irradiation. From the following figures, however, and from Table I it will be seen that such increase as took place in the CaO retention during irradiation is largely due to a decreased excretion in the faeces. Thus the average daily excretion of CaO in the faeces was 11·77, 11·53 and 11·69 g. in the pre-period, the irradiation period and the post-period respectively.

Experiment 2.

This experiment was carried out on the black and white goat at a time when she was in fairly full milk, yielding about 600 cc. per day. The experiment was divided into the following periods: (1) 5 days in inside pen; (2) 12 days pre-period; (3) 21 days irradiation, 4 hours per day; (4) 21 days post-period. Collections and analyses were carried out every third day.

Diet.

Food	Weight in g.	% CaO	% P_2O_5
Hay	900	0·9365	0·4560
Oatmeal	300	0·0785	1·0283
Linseed meal	50	0·3889	0·8901
100 g. oatmeal			
Total g. per day	—	7·4103	7·9193

During the irradiation period the goat's appetite increased and the milk yield showed signs of falling off. It was therefore necessary to increase the

[1] Throughout our irradiation experiments we have used these 10 ampere carbon arc lamps. In a previous paper [Henderson, 1925] the current consumption of the arc is wrongly stated [see Mayerson, Gunther and Laurens, 1926].

diet by 100 g. oatmeal per day after the second collection of the light period. Whilst such alteration of the diet is not entirely satisfactory any superimposed influence is probably small. A consideration of the excretion/intake ratio shows a definitely increased absorption of CaO and P_2O_5 during the irradiation period. Thus for CaO the ratios are: pre-period 1·04; light period 0·97; post-period 1·04. The corresponding ratios for P_2O_5 are 1·12, 1·00, 1·04. The decrease in faecal excretion of CaO during irradiation is seen from the following: pre-period 5·56 g. per day; irradiation period 5·39 g. per day; post-period 6·04 g. per day (see also Table I).

The composition of the urine showed little variation throughout the experiment. During the latter half of the irradiation period the milk yield increased, but this was probably due, at least partially, to the increased protein of the diet.

Experiment 3.

The same animal was used as in Exp. 2. In the present experiment, however, she was over 2 months pregnant when the experiment began, and was yielding only about 150 cc. milk per day. The periods of the experiment were as follows: (1) 12 days in inside pen; (2) 16 days pre-period; (3) 16 days irradiation period; (4) 24 days post-period.

Diet. This was identical with Exp. 2 except as regards the composition of the hay which was in the present instance slightly higher in calcium, *i.e.* 1·019 % CaO, 0·370 % P_2O_5. The total CaO fed per day was 9·69 g. and the P_2O_5 8·36 g.

Results. The balances are summarised in the graph and in Table II. As will be seen from the faecal excretions of CaO and P_2O_5 during the three periods (see Tables I and II) there was again increased absorption during irradiation. Thus the faecal excretions of CaO in the pre-, the irradiation and the post-periods were respectively 8·68, 8·11 and 8·21 g. per day. The corresponding figures for P_2O_5 were 6·75, 5·80, 5·59. The total excretion/intake ratios show a similar trend. Thus for the three periods the ratios are: CaO 1·04, 1·00, 0·99; P_2O_5 0·91, 0·82, 0·81. It is noteworthy that these figures obtained despite the fact that the milk yield continued to rise during the experiment until it was finally about 500 cc. per day.

It will be noted that in this experiment the increased absorption of the irradiation period is continued into the post-period. This is probably due to the fact that during the post-period the goat was in the last quarter of pregnancy.

Experiment 4.

The calcium and phosphorus balances were again followed out in the animal employed in the last experiment, a month after the birth of the kid and at a time when the goat was giving a good milk yield of about 1000 cc. per day.

The periods of the experiment were as follows: (1) 10 days in inside pen; (2) 16 days pre-period; (3) 16 days irradiation period; (4) 16 days post-period. Collections and analyses were made every fourth day.

Diet.

Food	Weight in g.	% CaO	% P_2O_5
Hay	900	1·388	0·476
Oatmeal	100	0·089	1·097
Maize meal	300	0·017	0·758
Linseed meal	50	0·389	0·890
Total g. per day	1350	12·83	8·10

Results. Balance figures are shown in Table II and are represented graphically in Fig. 1. The following figures show that there was a decreased faecal excretion of CaO during the irradiation period: pre-period 8·00, irradiation period 7·85, post-period 8·85 g. per day. The corresponding figures for P_2O_5 excretion are 3·90, 4·20, 4·97 g. per day. The increased absorption, therefore, of calcium which took place during the irradiation period was followed in the post-irradiation period by an increased excretion. The excretion of P_2O_5, however, increased slightly during the irradiation period and continued to increase more rapidly when the irradiation ceased. The significance of these results will be discussed later.

Of the four experiments, in this one alone was any marked increase noted in the urinary excretion of CaO during the light period. Thus the urinary excretion of CaO in g. per 4 days was as follows: pre-period, 0·45, 0·67, 0·56, 0·58; irradiation period, 0·72, 0·91, 0·84, 0·85; post-period, 0·84, 0·67, 0·59, 0·65. We have previously drawn attention to a similar phenomenon in the irradiation of pigs [Henderson, 1925]. Throughout the present experiment the urinary excretion of phosphorus remained fairly constant at the low level characteristic of the ruminant.

Discussion of Results.

Viewing the results of the four experiments as a whole it will be seen that in all four cases the retention of calcium increases during the irradiation period and falls again during the post-period. The total retention is, of course, influenced by the volume of milk secreted during each period, and in many cases this volume varied considerably. We do not propose to deal with the variations in volume and the small variations in composition in this paper. The effect of such variations upon the total balance is eliminated if the excretion in the faeces is alone considered. Table I shows the percentage of the intake which was excreted in the faeces in the various periods of the four experiments.

From Table II it will be seen that in all four experiments the faecal excretion of calcium was lower in the irradiation periods than in either the pre- or post-periods.

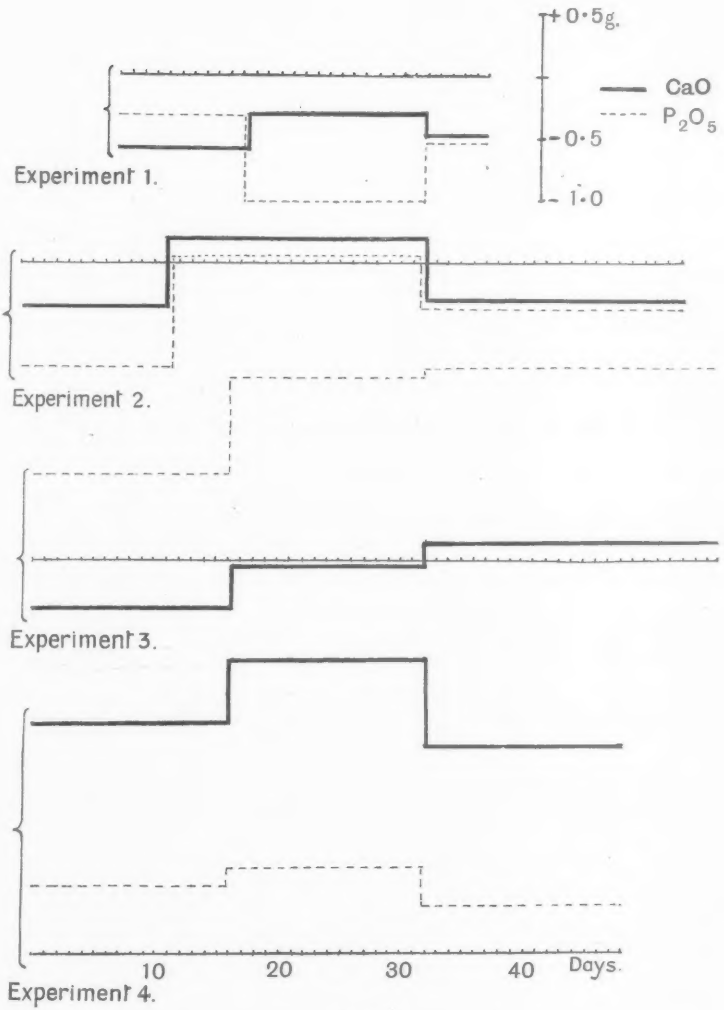

Fig. 1.

Graphs showing amounts of CaO and P_2O_5 in grams retained during the pre-, the irradiation and the post-periods of four experiments.

Table I. *% of CaO and P_2O_5 intake excreted in the faeces during each collection period.*

Exp. 1		Exp. 2		Exp. 3		Exp. 4	
CaO	P_2O_5	CaO	P_2O_5	CaO	P_2O_5	CaO	P_2O_5
101·8	95·3	81·3	74·2	105·6	92·4	66·5	55·2
97·9	87·5	84·6	93·8	78·0	70·8	62·9	46·1
101·3*	**98·9**	75·2	78·1	93·4	79·3	58·7	45·9
99·6	122·3	**76·9**	**76·0**	**101·1**	**57·2**	**61·3**	**47·3**
97·3	94·6	74·3	63·6	96·1	82·8	60·0	47·3
99·2	120·4	59·1	57·2	86·1	61·6	62·1	58·2
96·3	**94·1**	75·3	61·8	83·2	65·4	64·7	54·2
99·1	100·1	76·6	67·9	**90·5**	**75·3**	**58·2**	**48·4**
100·8	98·1	84·4	97·2	91·1	83·5	51·1	53·5
		70·1	70·5	79·8	67·4	57·5	67·2
		70·5	**57·7**	80·8	59·3	68·1	61·0
		80·5	74·4	89·3	65·2	99·1	66·8
		78·1	65·4	91·8	63·1		
		90·8	83·3	85·9	66·9		
		87·8	66·9				
		80·6	74·0				
		79·9	78·2				
		73·9	73·3				

* Heavy type indicates end of a period.

Table II. *Average daily intake, output and balance in g.*

	CaO					
	Intake	Output				Balance
		Milk	Urine	Faeces	Total	
Exp. **1.**						
Pre-period (11 days)	11·73	0·40	0·13	11·77	12·30	−0·57
Irradiation period (14 days)	11·73	0·35	0·13	11·53	12·01	−0·28
Post-period (5 days)	11·73	0·36	0·11	11·69	12·16	−0·43
Exp. **2.**						
Pre-period (12 days)	7·00	1·46	0·29	5·56	7·31	−0·31
Irradiation period (21 days)	7·38	1·50	0·29	5·39	7·18	+0·20
Post-period (21 days)	7·39	1·43	0·22	6·04	7·69	−0·30
Exp. **3.**						
Pre-period (16 days)	9·16	0·42	0·41	8·68	9·51	−0·35
Irradiation period (16 days)	9·13	0·62	0·42	8·11	9·15	−0·02
Post-period (24 days)	9·49	0·78	0·36	8·21	9·35	+0·14
Exp. **4.**						
Pre-period (16 days)	12·82	2·80	0·15	8·00	10·95	+1·87
Irradiation period (16 days)	12·82	2·35	0·21	7·85	10·41	+2·41
Post-period (16 days)	12·82	2·10	0·17	8·85	11·12	+1·70

	P_2O_5					
	Intake	Output				Balance
		Milk	Urine	Faeces	Total	
Exp. **1.**						
Pre-period (11 days)	5·10	0·55	0·05	4·80	5·40	−0·30
Irradiation period (14 days)	5·10	0·48	0·05	5·63	6·16	−1·06
Post-period (5 days)	5·10	0·52	0·04	5·10	5·66	−0·56
Exp. **2.**						
Pre-period (12 days)	6·72	2·08	0·06	5·41	7·55	−0·83
Irradiation period (21 days)	7·62	2·34	0·05	5·20	7·59	+0·03
Post-period (21 days)	7·91	2·34	0·07	5·83	8·24	−0·33
Exp. **3.**						
Pre-period (16 days)	8·18	0·69	0·04	6·75	7·45	+0·73
Irradiation period (16 days)	8·16	0·85	0·04	5·80	6·69	+1·47
Post-period (24 days)	8·26	1·06	0·05	5·59	6·70	+1·56
Exp. **4.**						
Pre-period (16 days)	8·09	3·61	0·03	3·90	7·54	+0·55
Irradiation period (16 days)	8·09	3·12	0·03	4·20	7·35	+0·74
Post-period (16 days)	8·09	2·60	0·03	4·97	7·60	+0·39

This relationship does not hold as regards the faecal phosphorus. In Exp. 1 its excretion is higher in the irradiation period than in either the pre- or post-period. In Exp. 2 the excretion is least in the irradiation period. In Exp. 3 the excretion of phosphorus in the faeces decreases progressively from the pre-period through the irradiation period to the post-period. Coupling this with the fact that in the post-period the faecal excretion of calcium is very little more than in the irradiation period we are inclined to attribute the tendency to a rising retention in Exp. 3 to the fact that the goat was pregnant. It would appear that irradiation gave an impetus to the natural process of mineral storage characteristic of pregnancy. One of us [Magee, 1924] has shown that just about this time (*i.e.* 14th week) the basal metabolic rate begins to rise rapidly in the pregnant goat. Hoffström [1910] found in experiments on pregnant women that the beginning of rapid growth of the foetus (in the 29th week) was accompanied by decreased excretion of calcium in the faeces of the mother. In Exp. 4 the faecal phosphorus gradually increased, this increase being most marked in the post-period. Considering this in conjunction with the marked increase in faecal excretion (1 g. per day) of CaO in the post-period it would appear that the goat was tending rapidly towards a negative balance, and that this tendency had been temporarily checked by irradiation. Despite the fact that the goat was in very good condition the initial positive balances of CaO and P_2O_5 in this experiment are unusual in view of the stage of lactation. We are unable to say to what extent they are contributed to by (1) the fact that following upon the post-period of Exp. 3 the goat was irradiated for 16 days (4 hours per day) in an investigation of the effect upon the composition of the milk, and (2) the quality of the hay. To our knowledge there had been no difference in the method of curing, but the calcium, phosphorus and nitrogen contents were all high.

As regards the calcium and phosphorus balances there is only a rough parallelism. Throughout all four experiments the phosphorus balances are less consistent. We have repeatedly noted in metabolism experiments on pigs that whilst the graphs of phosphorus and calcium retention may run parallel for a time the phosphorus curve occasionally, for no obvious reason, shows an unexpected transitory rise or dip. Forbes *et al.* [1922] in their metabolic experiments on cows draw attention to the "partial independence" of calcium and phosphorus.

In our work on pigs we [Orr, Magee and Henderson, 1924] noted that decreased faecal excretion of calcium or phosphorus in the irradiation period may be accompanied by an increased urinary excretion. Orr, Holt, Wilkins and Boone [1923] had found a similar effect in the irradiation of rachitic children; Mayerson, Gunther and Laurens [1925] have observed an increased urinary excretion of calcium and phosphorus on irradiating normal dogs. The figures of Hart, Steenbock and Elvehjem [1924] suggest a like relationship between faecal and urinary excretion in irradiated goats. In our present experiments the only point at which this effect is clearly brought out is the

calcium excretion of Exp. 4. Here the decreased faecal excretion of calcium in the irradiation period is accompanied by a corresponding rise in the urinary excretion of calcium. It is noteworthy that of the four experiments number 4 is the only one in which the calcium balance is positive throughout the experiment.

The results of Hart, Steenbock and Elvehjem [1924] and of the present experiments which indicate that the calcium, and to some extent, phosphorus retention during lactation may be definitely increased by ultra-violet irradiation suggest important practical applications. Whilst the negative balances of the first half of lactation may be largely physiological they are no doubt partially an adaptation to the artificial conditions of domestic milking animals. They probably, therefore, border closely upon the pathological, particularly in heavy milkers. Forbes and his associates [1922] have, for instance, pointed out that forced milk production in cows results in a loss of breeding capacity. Hart and his colleagues [1921, 1922, 1, 2] have observed "disaster in reproduction with cows where probably negative calcium balances are long continued." Orr [1923], moreover, has suggested that there is probably a relationship between high milk yield and susceptibility to tuberculosis. Lastly, Fingerling [1911] has shown that reduction of calcium and phosphorus in the diet of milking goats eventually causes the milk yield to fall. The last two points receive some support from a recent experiment carried out at this Institute by Orr, Crichton and Middleton [1925]. A group of heavy milking cows had calcium carbonate, sodium chloride, iron oxide and potassium iodide added to their rations. The amounts of the salts fed were determined by the milk yield of the cows. Another group of cows on the basal ration alone was used as controls. During the first lactation little difference was observed between the groups. In the second lactation the "mineral" group increased in milk yield whilst the yield of the "non-mineral" group diminished. Further, whilst several of the "non-mineral" cows reacted positive to the tuberculin test those of the "mineral" group were all negative.

In general, therefore, there seem some grounds for concluding that depletion of minerals in lactating animals may adversely affect breeding and milk production and may increase susceptibility to tuberculosis. Further, whilst in minimising such depletion the composition of the diet is of prime importance, ultra-violet irradiation would seem to be a valuable adjuvant.

Summary.

(1) In four balance experiments on lactating goats, irradiation by the carbon arc lamp reduced the faecal excretion of calcium. The effect upon the excretion of phosphorus was less constant. It seems probable that irradiation increases the stimulus to calcium storage during pregnancy and diminishes the calcium loss during full lactation.

(2) A definite increase in the urinary excretion of calcium on irradiation was only noted in Exp. 4 in which there was already retention of calcium.

(3) The influence of ultra-violet light in minimising the depletion of minerals is briefly considered with respect to certain pathological conditions which may occur in heavy milking animals.

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XLIX. EXPERIMENTS ON THE CHEMICAL NATURE OF THE OXYTOCIC PRINCIPLE OF THE PITUITARY GLAND.

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In spite of much work on the subject, very little is known of the chemistry of the oxytocic, or plain-muscle stimulating, principle of the pituitary gland. The great difficulty attached to attempts to isolate it as a pure chemical substance has been demonstrated by the work of Abel [Abel and Kubota, 1919; Abel and Nagayama, 1920] and of Dale and Dudley [Dudley, 1919; Dale and Dudley, 1921]. The most active fractions they obtained were mixtures of which probably only a small part was the actual active material, although their action was detectable in dilutions of one part in one to ten thousand millions. The principle being very sensitive to chemical reagents, the possible methods of purification are very limited. If, for example, it is precipitated by phosphotungstic acid, only a small fraction can be recovered from the precipitate. It is reasonably stable to acids but is destroyed by alkalis very rapidly even at room temperature.

In view of these difficulties the possibility of isolating the principle as a pure substance seems to be very remote and any information which can be obtained as to the chemical nature of the material is of value.

Its behaviour with alkali—it is inactivated on standing at room temperature in *N* sodium hydroxide for two hours—suggests that it might be an easily hydrolysable ester, whilst the ease with which it is destroyed by ordinary trypsin preparations would support the view that it might be a peptide, or at any rate have a peptide linkage in its molecule.

Now most preparations of trypsin contain also pancreatic lipase so that the inactivation of the principle by such ordinary preparations might be due, not to the peptide-splitting enzyme, but to the ester-hydrolysing lipase. If this were the case the hypothesis that the oxytocic principle contains a peptide linkage would be rendered less probable, whilst the view that it is an ester would receive strong support.

It was in order to obtain evidence as to whether this destruction by enzyme action was in fact due to trypsin or lipase, that the work reported here was undertaken[1].

[1] Dudley (unpublished experiments) had previously found that lipase from castor beans had no destructive action on the oxytocic principle, but it was considered highly desirable to make experiments with a lipase of animal origin.

For this purpose it was necessary to prepare either lipase-free trypsin or trypsin-free lipase. It was found that the lipase in dried pancreas could be easily destroyed by long standing in dilute acetic acid. From the extract a stable soluble powder was obtained which was strongly proteoclastic and had no lipoclastic activity.

The oxytocic principle of the pituitary gland was rapidly destroyed by this lipase-free trypsin preparation. The rate of inactivation produced by this preparation was compared with that produced by a lipase-containing preparation which had been obtained by the same method as the lipase-free preparation, except that the acetic acid was only allowed to act upon the dried pancreas for a short time. The tryptic activity of the two preparations was compared by two different methods giving different results, and quantities of the preparations, matched with respect to tryptic activity, were allowed to act upon the oxytocic principle. It was found that the lipase-free trypsin destroyed the principle much more rapidly than the lipase-containing preparation when quantities of trypsin, matched on the basis of either of the methods of estimation, were used. An investigation of this apparent retarding action of either lipase or some substance destroyed along with it was beyond the scope of this paper.

It is safe, however, to conclude from these experiments that pancreatic lipase plays no part in the inactivation of the plain-muscle stimulating principle of the pituitary gland and therefore that no support is gained for the view that it may contain an ester-like linkage. Its destruction by trypsin preparations appears to be due to the proteoclastic enzyme and therefore it is highly probable that the hormone possesses a peptide-like linkage.

Experimental.

Preparation of lipase-free trypsin. The starting material for the preparation was fresh pig's pancreas which was dried according to the directions of Willstätter and Waldschmidt-Leitz [1923]. Only that portion of the ground material passing a 40-mesh sieve was used for further treatment.

5 g. of this powder were left to stand with 100 cc. 0·1 % acetic acid for 5 days at room temperature. The acetic acid extract was separated from the residue by centrifuging and poured into a litre of 95 % alcohol. On standing overnight a white precipitate settled and was collected by centrifuging after syphoning off the main bulk of the alcohol. The solid was stirred up with acetone and centrifuged after standing half an hour. The powder was then dried *in vacuo* over sulphuric acid. The yield was 0·9 g. of a soluble and stable powder which had no lipoclastic activity. The trypsin content was much greater than that of the original dried pancreas and of ordinary trypsin preparations. Digestion with starch and testing with iodine showed that amylase was present. This lipase-free trypsin is henceforth described as preparation T.

For purposes of comparison another preparation, treated for a very short time with acetic acid, was made, which consequently still retained consider-

able lipoclastic activity in addition to its tryptic activity. This lipase-containing preparation is described as preparation L.

Estimation of tryptic activity. Two methods were employed for the estimation of the tryptic activity of the preparations. In one method the amount of substrate changed in a given time was determined and for this purpose caseinogen was used as the substrate. By the other method the time required to produce a given change in substrate was determined, milk being the substrate employed.

In the first method 20 mg. of the enzyme preparation were digested with 50 cc. 4 % caseinogen solution in 0·4 % sodium carbonate for 17 hours at 37°. Before and after digestion 20 cc. were withdrawn, run into 25 cc. neutral formaldehyde (13 %) and titrated against $N/10$ sodium hydroxide, using phenolphthalein as indicator.

Figures given for tryptic activity as estimated by the caseinogen method represent the increase in acidity of the substrate—that is, the difference of the two titration values after digestion under these conditions—and were obtained by comparison with the same sample of dried pancreas throughout.

Preparations T and L compared by this method gave the values:

Lipase-free preparation T	...	13·5
Lipase-containing preparation L		11·8

Thus, by this method of comparison, 5 mg. of T should be equivalent to 5·7 mg. of L.

In the second method the time required to coagulate a calcified milk solution was determined in accordance with the procedure of Mellanby and Woolley [1912]. Under the conditions described by these authors, a uniform curve was obtained with a commercial enzyme preparation and the preparations T and L employed in the pituitary experiments were compared in terms of the commercial preparation by reference to this curve. Several experiments using different strengths of the enzyme solutions all gave remarkably constant results, the activity of preparation T being related to that of L in the ratio 3·2 to 1. Thus, in this comparison, 5 mg. of T should match the tryptic activity of 16 mg. of L.

Estimation of lipoclastic activity. 5 g. of olive oil in 20 cc. water and 4 cc. 1 % diammonium hydrogen phosphate were adjusted to p_H 9·5 with $N/10$ sodium hydroxide (3 cc.) and well shaken for 3 minutes. The mixture was incubated with 20 mg. of enzyme for 17 hours at 37°. Before and after digestion 10 cc. were withdrawn, run into 50 cc. spirit and titrated against $N/10$ sodium hydroxide, using phenolphthalein as indicator. The figures given for lipoclastic activity represent the difference of the two titration values and were all obtained by comparison with the same sample of dried pancreas.

Preparations T and L under these conditions gave the values:

Preparation T	...	0·0
Preparation L	...	6·3

Action of acetic acid on dried pancreas powder. The figures given below show the action of 0·1 % acetic acid on the enzymes of dried pancreas at room temperature. It is seen that whilst the lipase is gradually destroyed, the tryptic activity is slightly increased, possibly owing to an activation of trypsinogen. Considerable activity was still present in the residue from the extractions.

Duration of acetic acid treatment of dried pancreas	Substrates	
	Caseinogen	Olive oil
Hours 0	5·0	5·0
2	10·1	6·6
48	12·1	2·6
72	13·8	0·5
96	14·1	0·1
140	13·4	0·0

Action of alcohol on the acetic acid extract. The following values were obtained to show the effect of precipitating the enzymes from the acetic acid extract by means of alcohol. They show that the trypsin content is only slightly decreased by standing overnight in alcohol. The acetic acid extract employed for this experiment had stood with the dried pancreas for 2 hours and was poured into the usual volume of alcohol.

Duration of alcohol treatment	Substrates	
	Caseinogen	Olive oil
2 hours at 20°	10·1	6·6
24 „ 37°	9·5	3·8
120 „ 37°	8·5	3·9

Action of the trypsin preparations on pituitary extract. The oxytocic principle of the pituitary extract was tested on the isolated uterus of the virgin guinea-pig in the usual way.

For the digestion the substrate was made up to match the p_H of 4 % caseinogen in 0·4 % sodium carbonate (8·5–9). 2 cc. of "Infundin" solution (Burroughs, Wellcome & Co.), 1 cc. water and 2 cc. $N/10$ sodium carbonate were added to the enzyme in 5 cc. 0·2 % sodium carbonate and incubated at 37°. At intervals 1 cc. was withdrawn and diluted to 20 cc. with 0·9 % saline. From 0·5 to 1 cc. of this solution usually gave the maximal dose for the uterus. A control containing the same quantity of boiled enzyme was employed throughout the experiments.

5 mg. of the preparation T rapidly destroyed the oxytocic principle of the pituitary gland. After 1½ hours' digestion the original maximal dose failed to produce a contraction and even four times this dose did not produce a maximal contraction of the uterus.

With a view to demonstrating that lipase was not able to cause the inactivation of the principle, the actions of the preparations T and L were compared side by side.

In one experiment the tryptic activity was matched on the basis of the caseinogen estimation, 5 mg. of preparation T and 5·7 mg. of L being used. Under these conditions preparation T destroyed the activity much more

rapidly than L. After 3 hours' digestion the original maximal dose of L produced a contraction of the uterus only slightly below the maximal, whereas it required six times this dose of T to produce half the maximal contraction.

When the two trypsin preparations were compared in detail on caseinogen analogous results were obtained, the initial rate of L being considerably slower than that of T. In fact curves drawn from values obtained with 30 mg. L and with 10 mg. of T intersect after 9 hours' digestion, 10 mg. of T showing more rapid proteoclastic activity during the first 9 hours. It was found impossible to obtain any consistent comparison of T and L by the caseinogen method even when the time required to produce a given change in substrate was considered. Consistent results could only be obtained when different quantities of the *same* trypsin preparation were compared. The curves obtained from T and L were of entirely different shape.

The action of the two trypsin preparations was then compared on the basis of the values obtained for tryptic activity by the milk coagulation method, 5 mg. of T and 16 mg. of L being used. Here also the lipase-free preparation was much more active. After $1\frac{1}{2}$ hours' digestion the maximal control dose of T failed to produce a contraction of the uterus, whereas the same dose of L gave a contraction only slightly below maximal. After 3 hours' digestion 5 cc. of T produced a contraction equal to between 2 and 3 cc. of L. Both contractions were below the maximal which was by this time produced by 0·3 cc. of the control solution.

Other work has, for the time being, prevented closer investigation of these trypsin preparations, but the experiments described leave no doubt that lipase does not participate in the destruction of the oxytocic principle by preparations of trypsin.

Summary.

1. The destruction of the oxytocic principle of the posterior lobe of the pituitary gland by ordinary "trypsin" preparations is due in fact to the trypsin and not to the lipase which they contain. This supports the hypothesis that the principle contains a peptide linkage in its molecule.

2. A simple method of making a lipase-free trypsin preparation is described.

During this work I have been greatly indebted to Dr H. W. Dudley for much valuable help and advice.

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L. STUDIES UPON CALCIFICATION *IN VITRO.*

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THE object of these experiments was to determine the conditions under which calcification occurs *in vitro*, and also what factors interfere with calcification. If it can be shown that the position and the character of the calcification, in other words the histological picture, are exactly the same as those occurring *in vivo* then it would seem justifiable to draw certain deductions regarding *in vivo* calcification from *in vitro* experiments. *Per contra*, if certain factors prevent calcification *in vitro*, and if it can be shown that these same factors are operative in diseases such as rickets, in which there is a failure of calcification, then it would appear justifiable to explain the failure of calcification by the influence of these factors.

The basis for these experiments was the observation of Shipley [1924] that, when the cartilage and bone of a rachitic rat are placed in the serum of rachitic rats and incubated for a number of hours, no calcification occurs. On the other hand, when a piece of cartilage and bone from the same animal is placed in the serum of normal rats and incubated for the same length of time, calcification, similar to that taking place in an animal undergoing cure, can be demonstrated. It is obvious that this method is applicable to the study of various aspects of calcification and for this it has been employed.

In previous studies of rickets from this department it has been shown that, in uncomplicated rickets, there is regularly a low concentration of inorganic phosphorus in the serum, 1·5 to 3·5 mg. per 100 cc., as opposed to the normal of 5 to 6 mg. Studies in conjunction with the School of Hygiene have demonstrated that rats can be rendered rachitic by feeding them a diet very low in calcium and relatively high in phosphorus or high in calcium and low in phosphorus (McCollum's 3143 diet). The low phosphorus diet is the most satisfactory one to use, for it produces a very severe rickets with great regularity. The low phosphorus in the diet is reflected in the low inorganic phosphorus of the serum and, instead of the normal 8 mg. of inorganic phosphorus in the serum, the rachitic rat has usually 2·5 to 3 mg. per 100 cc. It has further

been shown that, when as the result of some therapeutic procedure the inorganic phosphorus concentration of the serum rises, evidences of reparative processes appear in the bones. The position and the character of these reparative processes are perfectly definite and regular.

The information thus available determined the conditions under which we carried out the experiments especially as regards the concentration of the substances in the solutions employed. Certain precautions must be rigidly observed. The rats must be completely rachitic, *i.e.* there must be no trace whatever of calcification in the epiphyseal cartilages. Before utilising the bones of an animal we have stained a slice of bone and cartilage in order to determine freedom from calcification. If any calcification was present the animal was rejected. The animals must eat well up to the time they are killed. It has been shown that, if rachitic animals are starved for a period, the inorganic phosphorus of the serum rises and calcification of the cartilage begins. In some of the experiments of this series it has seemed that, if animals were deprived of food even for a few hours, the cartilage calcified more readily than does that of an animal which has been well fed. The animals must be killed without anaesthesia, by a blow on the head. The slices of tissue must not be too thin for reasons that will appear later. Aseptic precautions must be observed throughout the experiment.

Our procedure has been as follows. After the animal was killed, the skin was removed from a leg and with a sterile, small knife the muscles were removed and the knee joint disarticulated. The upper end of the tibia was isolated and slices about 0·5 to 0·75 mm. thick containing the centre of ossification, the epiphyseal cartilage, the metaphysis and a little of the shaft were made. One slice was examined for the presence of calcification. If satisfactory, the other slices from the same bone were immersed in solutions in 25 cc. Erlenmeyer flasks. The solutions had been previously prepared and warmed to 37°. After adding the tissue the flasks were immediately placed in an incubator and maintained at 37°. The solutions were made up in a number of different ways but all of them contained about 0·03 % sodium bicarbonate, $N/10$ NaCl and 0·0016 N $MgSO_4$. The calcium was varied from 0·0025 to 0·005 N (5·0 to 10·0 mg. per 100 cc.) and the inorganic phosphorus from 0·0015 to 0·009 N (1·5 to 9·0 mg. per 100 cc.). To each litre of solution 20 cc. of a 0·03 % sol. of phenolsulphonephthalein were added as an indicator. Certain solutions contained egg-albumin and some a colloid, gum arabic. The calcium salt in most of the solutions was the chloride, but the lactate, acetate and citrate were also employed.

All the components save the calcium were added and the solution was charged with carbon dioxide. The calcium salt was then added and water to the desired volume. The solution was filtered through a Berkefeld candle and the p_H adjusted by equilibration with CO_2 to reactions which were approximately physiological. The solutions were warmed, poured into 25 cc. sterile Erlenmeyer flasks and incubated for a few hours before use. No anti-

septics were used except when it was desired to study the effect of these upon calcification. The specimens were examined, usually at the end of 8 hours, and at intervals thereafter, sometimes as late as 2 weeks. At this time the p_H of the solution was read by comparison with phosphate standards and the temperature was determined. The tissue was removed, washed with distilled water, immersed in a 1 % solution of silver nitrate and examined under a binocular microscope with direct illumination.

When aqueous solutions were used we have sometimes set a time limit of 9 hours for the first examination. If at the end of this time calcification had not occurred we have considered the experiment negative. But this is purely arbitrary. In experiments with a normal calcium concentration (10 mg. per 100 cc.) and with a low concentration of phosphorus, calcification will occasionally occur at the end of 20 to 48 hours but with no regularity and the calcification is slight. Calcification regularly occurs in 9 hours when the concentrations of calcium and of inorganic phosphorus are 10 and 4 mg. respectively per 100 cc. This is shown in Table I.

Table I. *Calcification in aqueous solutions of inorganic salts.*

Ca mg. per 100 cc.	P mg. per 100 cc.	Precipitate	Calcification	p_H	Temp. ° C.	Time hours
10	1·0	0	0	7·42	35	9
10	2·0	0	0	7·35	35	9
10	3·0	0	0	7·45	35	9
10	4·0	0	+ + +	7·42	35	9

When the concentration of phosphorus is 3·5 mg. it requires 16 to 24 hours for a similar degree of calcification to take place. It is interesting to note that the result is the same whether the calcium is 10 mg. and the phosphorus 3·5 or the calcium 5 and the phosphorus 7 (Table II). The same process occurs with more concentrated solutions and very rapidly. We have obtained it with 9 mg. of phosphorus.

Calcification occurs best when no precipitation whatever takes place in the flask, for the obvious reason that precipitation lowers the concentration of calcium and phosphorus. Experiments are best made, therefore, at a p_H of 7·1 to 7·3 but the calcification will occur through even a wider range of reaction (Table II).

Table II. *Effect of reaction on calcification.*

Ca mg. per 100 cc.	P mg. per 100 cc.	Precipitate	Calcification	p_H	Time hours
10·0	3·5	0	+ + + +	7·05	24
10·0	3·5	0	+ + + +	7·10	24
5·0	7·0	0	+ + + +	7·00	24
5·0	7·0	0	+ + + +	7·10	24
10·0	3·5	0	+ + +	7·48	24
10·0	3·5	0	+ + +	7·50	24

In the above-mentioned experiments calcium chloride was used. We have made similar observations using the lactate, acetate and citrate in the same concentration of calcium (10 mg.) and with a concentration of inorganic phosphorus of 6 mg. per 100 cc. With the acetate and lactate, calcification was as prompt and thorough in 9 hours as with the chloride. With the citrate there was none even at the end of 48 hours.

The process of calcification is completely inhibited by certain substances. Previous soaking in chloroform, ether, 95 % alcohol, potassium cyanide (10 %), formalin (10 %), or toluene, or previously boiling the piece of tissue prevents calcification. A few drops of toluene added to the fluid prevented calcification in one experiment. The process does not occur in the cold (ice box).

Having established the fact that calcification regularly occurs in 24 hours with a phosphorus concentration of 3·5 mg. and in 9 hours with a concentration of 4·0 mg., we studied the effect of the addition of protein (1 and 2 %) to the solution. The protein employed was egg-albumin (Merck's soluble) which contained neither calcium nor inorganic phosphorus in sufficient amount to influence the results. The experiments are recorded in Tables III and IV.

Table III. *Effect of protein* (1 %) *on calcification.*

Ca, 10 mg. per 100 cc.; P, 3·5 mg. per 100 cc.

Precipitate	Calcification	p_H	Temp. ° C.	Time hours	Albumin %
0	+ + +	7·40	36	16	0
0	+ + + +	7·40	37	24	0
0	+ + + +	7·36	36	48	0
0	0	7·40	37	16	1
0	0	7·38	37	24	1
0	0	7·30	36	48	1

Table IV. *Effect of protein* (1 *and* 2 %) *on calcification.*

Ca mg. per 100 cc.	P mg. per 100 cc.	Precipitate	Calcification	p_H	Temp. ° C.	Time hours	Albumin %
10	4·0	0	+ + +	7·30	36·5	19	0
10	4·0	0	+ + +	7·35	36·5	19	1
10	4·0	0	0	7·25	37·0	26	2
10	4·0	0	0	7·25	37·0	47	2
8	5·0	0	+ + + +	7·45	36·5	7	0
8	5·0	0	0	7·20	37·0	42	2

The albumin clearly exerted a retarding influence, which was more marked in the higher concentration. With a 2 % albumin concentration no calcification occurred in 47 hours when the calcium was 10·0 and the phosphorus 4·0 mg. per 100 cc. A 4 % solution of gum arabic also had a striking inhibitory effect. That this failure of calcification was not due to injury of the tissue was demonstrated by the fact that the tissue at the end of 24 hours could be removed, washed and placed in an aqueous solution of the same inorganic concentration, when calcification promptly occurred.

We have repeated Shipley's original observation but have used human serum from normal and rachitic children instead of rat's serum. Calcification occurred in 19 to 48 hours in normal or relatively normal serum whereas in the rachitic serum it did not occur in the same interval of time.

Table V. *Calcification* in vitro *with human serum.*

Ca mg. per 100 cc.	P mg. per 100 cc.	Calcification	Time hours
	Non-rachitic serum.		
10·4	5·2	+	19
10·0	4·3	+	19
9·8	4·4	+	19
7·8	6·5	+	48
10·0	5·0	+ + +	20
	Rachitic serum.		
10·6	3·2	0	48
10·0	2·5	0	48
10·5	3·7	0	19

We have had an opportunity to make observations with human cartilage from two cases, one in a child who died suddenly and unexpectedly while convalescent from scurvy, and the other in an infant with severe rachitic changes in the thorax. In each instance the rickets was unusually severe. Pieces of costochondral junctions were placed in a solution with a concentration of calcium and of inorganic phosphorus of 10·0 and 6·0 mg. per 100 cc. respectively. Calcification occurred in less than 18 hours and was extensive in all the eight specimens examined.

When healing occurs in a bone affected by rickets calcification may come about in either of two ways. If recalcification goes on in response to a powerful stimulus, lime salts are laid down in the cartilage and a new zone of provisional calcification is formed before there is histological evidence of new calcification in the rachitic metaphysis. On the other hand, under certain conditions provisional calcification of the cartilage may be retarded and calcium phosphate may be deposited in the cartilage and the metaphysis simultaneously. In some cases the metaphysis begins to calcify before calcification of the cartilage is complete and the former process may be well under way before the latter is begun. The above is true for the healing of rickets in man or in the rat.

Calcification *in vitro* proceeds in the first mentioned manner and the first event is the formation of a line of calcification in the proliferative cartilage. As is the case when rickets heals in man, the calcification does not begin uniformly throughout the provisional zone but in small areas separated from each other by uncalcified cartilage, which, as the process proceeds, becomes calcified in such a manner that the interrupted line of calcification becomes a continuous one (Plate I, fig. 1).

The calcium phosphate is deposited in the intercellular matrix and never in the cytoplasm of the cells themselves, just as is the case when healing

occurs *in vivo* in the rat or in the body of the child. As a result of the freedom of the cells from calcification the calcified matrix has the appearance of a honey-comb (Plate I, figs. 2 and 3). Calcification *in vivo* or *in vitro* does not extend through the matrix of the entire zone of proliferative cartilage but is limited to the shaftward portion (Plate I, figs. 2 and 3). It is sharply delineated on the epiphyseal side and terminates in a straight or gently curved line (Plate I, figs. 2 and 5). The irregular tongues of cartilage which dip down into the metaphysis also become calcified.

Calcification does not begin on the surface of the slice of tissue as one might expect *a priori*, but the first deposits of lime salts are in the depths of the cartilage and the process gradually extends to the surface. It is for this reason that it is unwise to have the slices of tissue too thin, for, if they are, the calcification may be irregular or nearly wanting. It appears as if the trauma of cutting interfered somewhat with the integrity of the cartilage.

Celloidin sections from pieces of cartilage and bone calcified *in vitro* have the identical appearance of sections of cartilage and bone calcified *in vivo*. The calcified matrix has a granular appearance but there are no deposits in or on the cells themselves, when the sections are properly made and stained (Plate I, figs. 4 and 5).

Numerous attempts have been made to produce calcification in pieces of dead cartilage. Among them may be mentioned those of Pfaundler [1904], Wells [1911], Freudenburg [1922], etc. These experiments were made with the object of proving by chemical analysis the impregnation of the tissues with lime salts and have, therefore, no relation to our own work. More recently Robison and his co-workers [Robison, 1923; Robison and Soames, 1924; Kay and Robison, 1924] state that they have produced calcification of cartilage and bone *in vitro* by the action of an esterase upon solutions of calcium and phosphorus in the form of calcium hexosemonophosphate and calcium glycerophosphate. The concentrations of calcium and phosphorus were much higher than those found in normal serum and the reaction far more alkaline than is believed to be possible in the human body (p_H 8·4 to 9·4). Robison obtained uniformly negative results with concentrations of calcium and phosphorus corresponding to those found in normal serum and also negative results with higher concentrations when no esterase was added.

The calcification that was present in our experiments with inorganic salts as well as those with the calcium salts of lactic and acetic acids did not resemble the calcification pictured in the article by Robison and Soames. In our experiments the deposition of calcium was sharply limited to the intercellular matrix between the cells of the provisional zone (Plate I, figs. 3 and 5). In many instances the intercellular matrix of this whole zone was completely calcified. There was no granular deposit in the cells or in the resting cartilage. A granular deposit was found on the surface of the slices a few times when the solution was strongly alkaline and a precipitate had formed in the solution, but when a precipitate had formed calcification was usually incomplete

or absent. In all the experiments that we have recorded in the beginning of this paper calcification was unaccompanied by precipitation.

Robison has suggested an ingenious theory to explain calcification. Briefly this theory is as follows. Calcium and phosphorus exist in the blood and presumably in the tissue fluids in part as calcium hexosemonophosphate, in part as tertiary calcium phosphate. By means of this distribution they are enabled to remain in solution in serum at their high, normal concentration. Normal as well as rachitic cartilage and bone and also other tissues contain an esterase which liberates inorganic phosphorus from its organic hexose combination. Tertiary calcium phosphate is formed in excess of its solubility and therefore precipitates. Before accepting this theory there are certain aspects of the whole problem that should be considered. Do the calcium and phosphorus of the serum exist to a significant amount as calcium hexosemonophosphate? Robison himself has been unable to answer this question in the affirmative. He has, however, demonstrated the presence in red blood cells of an organic phosphorus compound which liberates inorganic phosphorus upon the addition of esterase[1]. We observed several years ago that when the serum of normal or rachitic children or animals is allowed to stand at 37° in contact with the clot the inorganic phosphorus of the serum rapidly rises. The extent of this increase is shown in Table VI.

Table VI. *Effect on inorganic phosphorus of serum of standing in contact with clot.*

(Temperature 37°.)

	Mg. per 100 cc.				Mg. per 100 cc.		
	Before	After incubation	Time hours		Before	After incubation	Time hours
Rat	3·5	6·2	24	Infant	6·6	20·0	24
„	2·9	10·3	24	„	3·3	16·0	24
„	2·9	6·2	24	„	3·4	16·0	24
„	7·0	24·0	24	„	3·4	12·0	24
„	2·8	11·2	24	„	3·5	15·0	72
„	6·0	10·4	24	„	2·2	13·2	24
„	7·0	13·0	24	„	6·0	18·0	24

For this reason we have insisted upon the necessity of prompt separation of the serum. Obviously, therefore, the conversion of organically bound phosphorus and the diffusion of the latter from the cells into the plasma can occur *in vitro* even in the absence of esterase. We have shown that, if inorganic phosphorus and calcium are present in such concentrations as in normal serum, calcification promptly occurs without the addition of esterase other than that which might be present in the slice of tissue undergoing calcification. If esterase plays a determining rôle in calcification why is it present in large amounts in rachitic as well as in normal cartilage and in tissues that are the seat of calcification only under exceptional circumstances? We have found

[1] However, it is unlikely that the organic compound of the red blood corpuscles is a hexosemonophosphate, for the ratio of hexose to phosphorus in the barium salt that he isolated is not that which obtains in barium hexosemonophosphate.

that the immersion of slices of tissue in antiseptics or the use of these in the solutions definitely inhibits or entirely prevents calcification. Robison's experiments have been done with antiseptics such as chloroform. For this reason it does not seem remarkable to us that the calcification which Robison obtained should have been inconstant and incomplete.

The reaction (p_H) at which Robison's experiments were carried out (p_H 8·4–9·4) is one which can hardly be considered to obtain in the body and in our own experiments has been found to interfere with, rather than to facilitate, calcification.

The illustrations in the article by Robison and Soames resemble the diffuse deposition of calcium phosphate from very alkaline solutions rather than the calcification which takes place *in vivo* in the process of healing. Granular deposits over the cells themselves may be formed during the process of staining sections if this technique is improperly carried out.

The fact that *in vitro* calcification occurs in simple solutions of inorganic salts including calcium and phosphate, with or without protein, at a temperature and reaction and in concentrations of calcium and phosphorus which may be supposed to exist in the animal body, and that this calcification is similar in all respects to the calcification which occurs *in vivo*, makes it unnecessary to assume the local action of an esterase upon a substrate of calcium hexosemonophosphate. In fact it is unlikely that such an action can play a rôle in calcification since the calcium and phosphate which reach the cartilage cells already exist in sufficient concentration for calcification in inorganic form. This is true in the serum and presumably the tissue fluids of normal children. In rachitic children the concentrations are insufficient.

It is not to be denied that such a process as Robison describes might explain the liberation of inorganic phosphorus from the red blood cells but there seems to be no special necessity for assuming that this takes place almost entirely in the bones and cartilage, or to postulate the necessity of an enzyme to bring this about since it can be shown that under conditions identical with those described by Robison liberation of inorganic phosphorus from the corpuscles occurs, without the addition of enzyme.

Lehnerdt [1910] in his excellent article upon the failure of calcification in rachitic bones came to the conclusion that this failure was due, not to a primary deficiency of calcium in the diet or to an abnormal metabolism producing an increased excretion of calcium but to an inability of the newly formed bone to incorporate into itself the circulating calcium. It is obvious that Lehnerdt, and many others, regarded the primary disturbance as one chiefly associated with calcium. The fact that the calcium concentration of the serum in uncomplicated rickets is often quite normal or reduced only to a slight extent, whereas the inorganic phosphorus concentration is regularly much lower than normal, indicates that the primary disturbance concerns the phosphorus.

It has been shown by the experiments recorded above that the bones and

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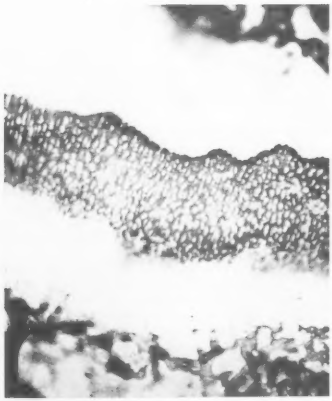

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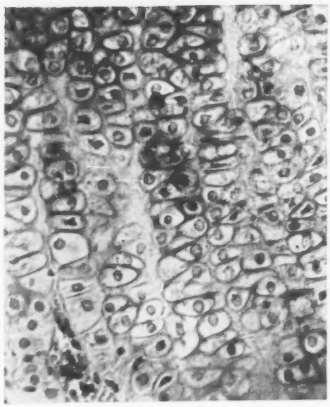

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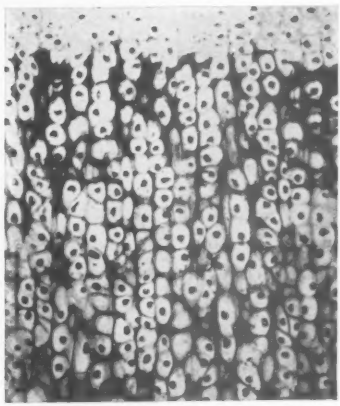

5

cartilage are perfectly capable of undergoing calcification provided the concentrations of phosphorus and calcium are adequate, whether in simple inorganic solutions, in solutions with albumin, or in relatively normal serum. They will not undergo calcification if the concentration of inorganic elements is insufficient. The conclusion is inevitable that the disturbance is not primarily in the bones. They do not calcify because the substances necessary for this do not reach them in sufficient concentration.

SUMMARY.

Calcification occurs *in vitro* apparently exactly as it does *in vivo*. In aqueous solutions it occurs rather rapidly only when the concentration of the bone-forming constituents and the reaction are nearly those of normal serum. Calcification is delayed by the addition of egg-albumin and completely inhibited by heat, by cold and by protoplasmic poisons.

It is impossible to advance any theory to explain the selective deposition of the calcium salts. The process is clearly not one of simple precipitation. It depends upon the activity of living tissue. It cannot occur unless the concentration of calcium and phosphorus in the serum and presumably in the fluid bathing the cells exceeds a certain minimal value.

The failure of calcification in rickets does not depend upon any primary inability of rachitic bone or cartilage to undergo calcification. The failure depends upon an insufficient concentration of the necessary elements in the fluids bathing these tissues.

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DESCRIPTION OF PLATE I.

Fig. 1. Proximal end of the tibia removed from a rachitic rat and kept for 19 hours in a solution of salts containing 10 mg. of calcium and 4 mg. of inorganic phosphorus per 100 cc. The newly deposited lime salts were blackened with silver nitrate. × 8.

Fig. 2. Photomicrograph of a part of the newly formed provisional zone of calcification. The specimen from which this was taken had been dehydrated and cleared in oil of wintergreen after treatment with nitrate of silver. × 85.

Fig. 3. This and the above figure show the honeycomb-like appearance of the provisional zone of calcification. This figure, taken from a slice of bone prepared as described above, shows that the calcification is confined to the intercellular matrix and does not involve the cell bodies. × 400.

Figs. 4 and 5. These photographs were made from celloidin sections (stained H. and E. and silver nitrate) of slices of bone taken from a costochondral junction of a rachitic child who had died 18 hours before *post mortem* examination. The tissue photographed for Fig. 5 had been kept 24 hours in a solution of salts containing calcium 10 mg. and inorganic phosphorus 4 mg. per 100 cc. The blackened deposits of lime salts are in the cartilage matrix between the cells. Fig. 4 was taken from a specimen fixed at autopsy and shows the cartilage without a trace of calcification. × 350.

LI. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

A REPLY TO SHIPLEY, KRAMER AND HOWLAND.

By ROBERT ROBISON.

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(*Received January 18th, 1926.*)

In a paper which appears in the present number of this *Journal*, Shipley, Kramer and Howland [1926] give an account of experiments in which calcification was found to occur in slices of cartilage and bones of rachitic animals when these were kept *in vitro* in normal serum or in aqueous solutions containing calcium salts and inorganic phosphates.

Arguing from these very interesting results they reject the hypothesis [Robison, 1923] that calcification depends on the activity of an enzyme secreted by the osteoblasts and hypertrophic cartilage cells. A final decision on this question cannot be arrived at without further investigation, but I wish to deal very briefly with some of their remarks which appear to be based on misapprehension with regard to the experimental evidence on which the hypothesis rests.

The brief description of the theory on p. 385 does not correctly represent my statements. Although tertiary calcium phosphate (or a more basic salt) is deposited from the tissue fluids, the amount of this salt existing in solution in these fluids and in blood constitutes an exceedingly small fraction of the total inorganic phosphate, most of which occurs as HPO_4'' and H_2PO_4' ions.

On p. 384 the following statement appears: "Robison obtained uniformly negative results with concentrations of calcium and phosphorus corresponding to those found in normal serum and also negative results with higher concentrations *when no esterase was added.*" *No esterase was added* in any one of these experiments, the whole purpose of which was to demonstrate the pre-existence and the precise location of the enzyme in the tissue itself. It is quite true that the concentration of calcium in the solutions of calcium glycerophosphate was much higher than it is in blood, but these solutions contained no inorganic phosphate at all. It was not claimed that the conditions represented those found *in vivo* or that the results were exact histological pictures of normal calcification. But it was claimed that a bone when supplied with calcium and with a suitable phosphoric ester can, by its own enzymic activity, hydrolyse this ester and deposit the inorganic phosphate thus produced in the form of calcium phosphate, and further that this deposition takes place in precisely those regions in which normal calcification would have occurred.

The criticism on p. 386 that "the illustrations resemble the diffuse deposition of calcium phosphate from very alkaline solutions" misses the obvious fact that without the action of the enzyme no inorganic phosphate existed and therefore none could be deposited. Their suggestion that the deposit may have been due to faulty staining may be passed over without comment.

Again on p. 386 the remark "there seems to be no special necessity...to postulate the necessity of an enzyme" indicates some misapprehension of the facts. The presence of the enzyme in bone and in ossifying cartilage has not been postulated but experimentally proved and its complete absence from cartilage in the early stages of bone development, prior to the beginning of ossification, has also been proved [Martland and Robison, 1924, 2].

Page 385. The presence of calcium hexosemonophosphate in the blood, however interesting from other aspects, is not in any way necessary to the enzyme theory of calcification. As I have clearly pointed out, the requirements of the scheme would be satisfied equally well by glycerophosphate or any other phosphoric ester which is hydrolysed by the bone enzyme and not by the muscle esterase. The presence of such an ester has been definitely proved [Kay and Robison, 1924] although its chemical nature has not yet been established. It is unnecessary to discuss here the question of its relationship with hexosemonophosphate, but with regard to the footnote on p. 385 it should be noted that the reducing power of hexosephosphoric esters is not the same as that of the free hexose but is always much lower [Robison, 1922].

In the next paragraphs there appears to be some confusion of ideas with regard to esterases. The authors state that when serum is allowed to stand in contact with its clot the conversion of organically bound phosphorus can occur "even in the absence of esterase." The increase of inorganic phosphate which occurs under such conditions has been recognised by many workers, and has been shown [Martland and Robison, 1924, 1; Martland, Hansmann and Robison, 1924; Martland, 1925] to be due to the action of a phosphoric esterase present in the corpuscles. This enzyme is quite distinct from that which occurs in bone. Its optimum p_H lies at or slightly below 7·0 and it hydrolyses those phosphoric esters of the blood upon which the bone enzyme has no action. The converse also holds. *The phosphoric esters of the blood are of more than one type and may serve more than one purpose.*

The occurrence of the enzyme in rachitic bones (p. 385) presents no difficulty, for a number of other factors besides the enzyme are necessarily involved in calcification and failure of any one of those would be enough to prevent the normal process from taking place [*v.* Robison and Soames, 1925]. It is also not difficult to understand the presence of the enzyme in the intestinal mucosa since it may serve for the hydrolysis of phosphoric esters present in foodstuffs.

Its function in the kidney has been the subject of recent investigation [Eichholtz, Robison and Brull, 1925] and evidence has been obtained that

the enzyme may play an important part in the normal secretion of inorganic phosphates.

The liberation of inorganic phosphate by the action of an esterase in any tissue will not necessarily be followed by the deposition of calcium phosphate in that tissue. Other factors such as the p_H of the tissue fluids may play a determining part.

The results obtained by Shipley, Kramer and Howland give a certain reasonableness to their argument that the intervention of a phosphoric ester and esterase is unnecessary in order that calcification should take place, but they are in no way opposed to the views which I have put forward, for it has not been suggested that the enzymic hydrolysis of the phosphoric ester is the sole mechanism in calcification. A possible supplementary mechanism has even been suggested [Robison and Soames, 1924], viz. that the osteoblasts and hypertrophic cartilage cells may have the power to raise the p_H of the tissue fluids in which they are bathed. The effect of this on a solution saturated with respect to $Ca_3(PO_4)_2$ and $CaCO_3$ would be to cause a precipitation of both these salts. This would account for the presence of calcium carbonate which forms 10 % of the inorganic constituents of bone, and, according to Bassett [1917], is precipitated separately and not as part of a complex carbonato-phosphate. At the same time the activity of the enzyme would be increased, thus greatly accelerating the precipitation of calcium phosphate over that of the carbonate. It is not necessary to assume that the p_H is raised to 8·4. A small upward change would produce an effect of the sort described. The results obtained by Shipley, Kramer and Howland could be satisfactorily explained on this assumption although they provide no proof of its correctness. It is not unreasonable to suppose that this mechanism would be destroyed by treatment with chloroform, cyanide, formalin, etc.

Unless it is accepted that the presence of enzymes in body tissues may be entirely fortuitous and have no significance whatever it is idle to argue that they cannot function in any particular way because their optimum p_H is impossible of attainment by the cell fluid. It would be more reasonable to enquire whether their optimum p_H (as determined *in vitro* with tissue extracts) may not be modified within the body[1]. One fact bearing on this question may be given. The optimum p_H of the enzyme in kidney extracts is the same as that in bone extracts but definite evidence of marked enzymic activity was obtained when surviving kidneys were perfused with blood at the normal p_H *whatever may have been the* p_H *of fluid within the kidney cells* [Eichholtz, Robison and Brull, 1925].

In the foregoing discussion, the results of Shipley, Kramer and Howland, in so far as they relate to the calcification of cartilage immersed in solutions of calcium salts and inorganic phosphates, have been accepted without

[1] The influence of endocrine secretions on the optimum p_H of liver diastase has been studied by Langfeldt [1921] who obtained evidence of positive effects with adrenaline and thyroiodine. Similar investigations on the bone enzyme by Martland and Robison and by Demuth [1925] have so far failed to detect any such influence.

question. It is obvious that the results obtained with normal serum fit in equally well either with their views or with my own, since the necessary phosphoric ester might be present in such serum. Their statement that in the experiments with aqueous solutions calcification occurred best "when the concentration of the bone-forming constituents and the reactions are nearly those of normal serum" requires further consideration.

It is probable that only about 53 % of the calcium in serum is diffusible through normal animal membranes [Cameron and Moorhouse, 1925] and that the percentage of ionised calcium[1] is still lower than this[2], whereas in aqueous solutions of calcium phosphate containing 10 mg. calcium per 100 cc. upwards of 80 % of the calcium is present in the form of its ions. It is the ionic concentration and not that of total Ca (and P) which determines the condition of supersaturation and eventual precipitation of solid salt, and in respect of calcium ions the solutions used by Shipley, Kramer and Howland were much more concentrated than normal serum. These solutions were supersaturated with respect to tertiary calcium phosphate and the results obtained appear to have depended largely on this condition of supersaturation, for the authors state "calcification occurs best when no precipitation whatever takes place for the obvious reason that precipitation lowers the concentration of calcium and phosphorus."

It is very suggestive also that in these experiments calcification was delayed or totally inhibited by the addition of 1 or 2 % of albumin to the solutions, a result which does not accord very well with the view that the factors determining the process in these aqueous solutions were exactly the same as those which operate *in vivo*. The effect of the protein can be readily understood if it is assumed that a sparingly dissociated calcium-protein compound is formed, since the concentration of calcium ions and the degree of supersaturation would thereby be reduced. The existence of such a compound probably accounts for at least part of the non-diffusible calcium in plasma.

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[1] Or the "activity coefficient" in the terminology of the modern theories of strong electrolytes.
[2] Neuhausen and Marshall [1922] from electrometric determinations conclude that 80 ± 5 % of the calcium in serum is present in some un-ionised form.

LII. RÔLE OF INSULIN IN PHOSPHORUS METABOLISM.

By SOPHIE KOŁODZIEJSKA and CASIMIR FUNK.

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(*Received February 2nd, 1926.*)

The work of Harden and Young, Lebedev and others has established the rôle of phosphoric acid in the process of fermentation of sugars. This viewpoint has been adapted by Embden and his school to the metabolism of sugar in animal tissues. As Embden expressed the opinion that the first stage of sugar utilisation is the formation of hexosephosphoric acid (lactacidogen), one could *a priori* accept that insulin administration would produce a marked disturbance of the phosphorus metabolism, especially in the equilibrium between the inorganic and organic phosphorus moiety. That this is really the case has been shown by several authors. Among these Wigglesworth, Woodrow, Smith and Winter [1923] have demonstrated that, on introduction of insulin into the animal organism, the inorganic phosphorus of the blood is suddenly lowered and is maintained at a low level for a considerable time, whereas the introduction of glucose causes the reverse process to occur. Similarly Sokhey and Allan [1924] have performed a series of experiments on the influence of insulin on the excretion of phosphorus in the urine. They have observed that in certain animals six hours after the insulin injection the inorganic phosphates diminished whilst in the subsequent stage a corresponding increase was noticed. The total phosphorus output calculated on a 24-hour basis was higher than normal to the extent of 25 to 35 %. Similar results were obtained by supplying sugar alone or insulin with sugar.

Based on these results we have thought for a long time [1925, 1, 2] that the lack of proportionality between small and high doses of insulin preparations, an observation which has been also described by Laqueur and de Jongh [1925], might be due to the quantity of phosphorus immediately available to the animal to effect the lactacidogen formation. The unequal activity of identical doses of insulin could, perhaps, also be explained on that basis. We must immediately state here that our experiments performed to test this hypothesis point to the minor part played by phosphorus in the insulin action and that the explanation of the mechanism of the reaction has to be thought of in a different direction. Our results show that whilst insulin may cause a shifting of the mode of occurrence of the phosphorus (inorganic into organic com-

bination or the reverse) it seems that it exerts a minor influence on the general phosphorus metabolism. To test the points in question we made first a whole series of experiments on the phosphorus distribution in the blood under the influence of insulin. The analytical results obtained (not recorded here) show such a lack of constancy (due probably to the various stages of lactacidogen formation and decomposition, owing perhaps to the particular stage of synthesis or decomposition which might be found in the animal at the time of bleeding) that we decided to attack the problem in a different way.

The experiments which were performed on rats can be divided as follows: (1) experiments on the excretion of phosphorus in the urine and faeces under the influence of insulin (and also vitamine B), on a diet containing phosphorus only in the form of caseinogen; (2) experiments on rats, showing the phosphorus metabolism under the influence of insulin and vitamine B on a phosphorus-free diet; (3) experiments on rats kept, previous to the administration of insulin, on a diet entirely devoid of phosphorus.

Experimental.

The first experiment was performed on four male rats, kept in special cages, which are described elsewhere [Funk, 1925], and which permit a quantitative separation of the excreta. The animals were fed on an artificial diet with a salt mixture free from phosphorus, prepared according to Pappenheimer, McCann and Zucker [1922]. The experiment lasted 16 days: two preliminary periods, one insulin period (2 doses, 4 units per kg.) and one vitamine B period. Here is the summarised result of the experiment.

Periods	Change in weight g.	P intake mg.	P in urine mg.	P in faeces mg.	Total P excretion mg.	P balance mg.
Preliminary	−3·5	31·05	4·41	26·78	30·94	−0·13
Insulin	−2·2	28·02	3·55	23·57	27·12	+0·9
Vitamine B	+2·2	33·69	2·57	29·83	32·42	+1·29

The second experiment was performed on a phosphorus-free diet (egg-albumin instead of caseinogen). 24 hour collections of urine and faeces were made on four animals continued for 34 days. Here follow the results.

Diet	Food intake g.	Change in weight g.	P in urine mg.	P in faeces mg.	Total P excretion mg.
P-free diet alone	5·0	−1·37	0·79	1·89	2·6
P-free diet and insulin	4·8	−1·93	1·09	1·70	2·68
P-free diet and vitamine B	7·4	+3·25	0·40	2·61	2·99

The third experiment was made on three groups of rats of four animals each, all kept on a phosphorus-free diet. The animals themselves were analysed for phosphorus. The first group of rats were killed on the tenth day of the phosphorus-free diet, the second group were injected with 4 units of insulin every third day and the third group were used as controls and were killed on the day on which the animals treated with insulin died.

Group of animals	Initial weight g.	Final weight g.	Average P content per rat g.	P %
I. Killed on the 10th day of the P-free diet	122	116	0·921	0·82
II. Insulinised animals	122	94	1·019	1·07
III. Controls	124	86	0·979	1·14

Results of these three experiments show that insulin exerts only an insignificant influence on the total phosphorus metabolism.

Summary.

1. The change in the phosphorus distribution of the blood under the influence of insulin does not give us the true picture of its influence on the total phosphorus metabolism.

2. The metabolism experiments on the phosphorus partition in the urine and faeces under the action of insulin on a diet containing phosphorus, as well as on a diet devoid of phosphorus, show that the total phosphorus metabolism remains practically unaffected.

3. The same conclusions can be reached if the animals are analysed *in toto* on a phosphorus-free diet under the influence of insulin. No difference in the phosphorus content of insulinised animals was found as compared with the controls.

4. Insulin seems to play an insignificant rôle in the total phosphorus metabolism.

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LIII. THE ACTION OF PURE PHOSPHATIDES ON THE PERFUSED HEART OF THE FROG.

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(*Received February 15th, 1926.*)

The study of the pharmacological properties of the phosphatides has until recently been hindered by lack of chemical criteria of purity, the result of our ignorance of the chemistry of these compounds. The work of Rosenheim, MacLean, Levene and others, chiefly in the last decade, has however brought us much nearer to an exact knowledge of their chemical constitution, and rendered it possible to use for biological experiments phosphatide preparations of a high degree of purity [see MacLean, 1918]. The object of the present work was to prepare samples of lecithin and kephalin of known purity and to test the influence of these substances on the perfused heart of the frog.

In considering criteria of purity it is necessary to survey briefly the present state of our knowledge of the chemistry of these compounds. Lecithin has been shown to have a molecular weight[1] of the order of 800 [Levene, 1921; Levene and Rolf, 1923]. It is a diglyceride, containing approximately equal proportions of saturated and unsaturated fatty acids. Of saturated acids only palmitic and stearic have been identified with certainty [Koch, 1902; Ulpiani, 1901; Cousin, 1906; Levene, 1921], but there are indications of several unsaturated acids [Levene, 1921; Levene and Rolf, 1923], of which oleic, linoleic and arachidonic have been confirmed. It appears then that a preparation of "pure" lecithin is a mixture of a series of lecithins with molecular weights ranging between 760 (palmityl-linolyl) and 810 (stearyl-arachidonyl), having the generic formula

Glycerol—< Saturated fatty acid / Unsaturated fatty acid / Phosphoric acid—Choline

There are possibilities of space isomerism, which need not be considered in this connection. A kephalin apparently differs from a lecithin in containing the base colamine in the place of choline [MacLean, 1915].

A lecithin preparation may therefore have a nitrogen content anywhere between 1·70 and 1·85 % and still be quite pure. Neither the nitrogen content nor the phosphorus content is any test of purity, though if the present theory

[1] Some as yet unpublished work by the writer has shown that kephalin has an equivalent weight of the same order of magnitude. The full titration curves of both phosphatides will be published shortly.

is correct the atomic ratio of these two elements must always be unity, if the preparation is pure. Any mixture of lecithin and kephalin may satisfy this criterion, but estimation of the amino-nitrogen content affords an easy control on the proportion of kephalin present.

For the purposes of this work a lecithin sample was considered pure when its nitrogen-phosphorus ratio was unity and it contained no amino-nitrogen, and in addition gave negative tests for cholesterol, galactose and mineral impurities. A kephalin sample was tested in the same manner, and had to contain no non-amino-nitrogen.

Preparation and Purification of Lecithin and Kephalin.

Preparations were made of lecithin and kephalin from egg-yolk and from ox-brain. The egg-yolks had been separated from the whites and frozen without preservatives and in the absence of air; the ox-brains were always fresh. The method of treatment was the same in each case. It was adapted from the methods used at various times by Rosenheim, MacLean and Levene. The tissue was minced and dried by extraction with acetone; it was then exhaustively extracted with dry acetone, absolute alcohol and sodium-dried ether in succession. These and all subsequent operations were performed in an atmosphere of nitrogen. The fat-free residue was rejected and the extracts treated as follows.

Combined acetone extracts. These were maintained at 0° till no more solids crystallised out. The deposit was repeatedly extracted with small quantities of warm dry alcohol, each extract being precipitated with alcoholic $CdCl_2$. These precipitates were mixed and subsequently purified.

Alcoholic extracts. These were precipitated at once with $CdCl_2$; all precipitates were mixed and subsequently purified.

Ether extracts. These were concentrated to half their bulk and crude kephalin precipitated by means of acetone.

Purification and decomposition of lecithin-$CdCl_2$. The use of toluene, or of ethyl or methyl acetate, was considered undesirable in view of the relatively powerful pharmacological activities of these substances. The only purification adopted was "crystallisation" from moist ether. The compound freezes out from moist ether in granular lumps when a certain degree of purity is attained. It was then decomposed by Bergell's method [1900]. This is admittedly the weakest part of the technique. It involves heating an aqueous-alcoholic solution of the compound to 70° while a saturated solution of ammonium carbonate is added until the mixture is alkaline, when it is filtered hot. These conditions are very favourable to destruction, but by working in an atmosphere of nitrogen, and freezing the filtrate instantly, the risk was minimised as far as possible. The solution was kept at − 10° for an hour and the liquor decanted; this mother-liquor was added to the crude lecithin-$CdCl_2$ extracts. It is not wise to add acetone to the alcoholic filtrate as lecithin is very soluble in acetone-alcohol mixtures.

Purification of the free phosphatides. Lecithin. This was repeatedly precipitated with acetone from ether solution; it was then emulsified with water and coagulated with acetone, this being repeated several times. It was then dissolved in ether and reprecipitated with acetone to remove water. This also was repeated a few times. Finally the pure lecithin was extracted with dry acetone for several days at 0° to remove the last traces of ether and water. At this stage the lecithin had the appearance of a waxy, finely granular solid, quite white. It was dried in nitrogen over H_2SO_4 after as much acetone as possible had been pressed out. The product has been kept sealed up in nitrogen at low pressure for 2 years without any apparent deterioration.

Kephalin. This was obtained from the ether extract of the brain powder by repeated solution in ether and gradual reprecipitation with alcohol. The product was then repeatedly emulsified with water and coagulated with alcohol (not with acetone: alcohol is more effective). After several more precipitations from ether solution the product was extracted with dry acetone at 0° and finally dried in the same manner as the lecithin. P_2O_5 was tried as a desiccating agent, but it appeared to induce polymerisation of the traces of acetone present, causing the phosphatide to darken.

Analytical data tabulated.

Preparation	% P	% N	$\frac{\text{amino-N}}{\text{total N}} \times 100$	N/P ratio
Lecithin (egg)	4·01	1·85	12	100 : 98
Lecithin (brain)	3·92	1·87	5	100 : 95
Kephalin (brain)	3·88	1·82	86	100 : 96
Pure lecithin (theoretical)	3·8–4·1	1·7 –1·85	0	100 : 100
Pure kephalin (theoretical)	4·1–4·4	1·85–2·00	100	100 : 100

The Pharmacological Action of the Phosphatides.

The results of injection and ingestion of "lipoids" have in most cases in the past been vitiated by the doubtful purity of the preparations used. In the present work results are submitted which indicate that one at least of the supposed properties of the phosphatides is non-existent, and that such effects as have been observed by previous workers have probably been due to water-soluble impurities in the preparations used.

Clark [1914] showed that crude lecithin (alcoholic extract of egg-yolk: Kahlbaum) had a strong stimulating action on the hypodynamic heart of the frog, and that a similar action was produced by alcoholic extracts of serum and by sodium oleate and various other soaps. Lecithin of this nature also produced a slight stimulant action on the isolated gut of the rabbit and on the isolated uterus of the guinea-pig [Clark, 1920]. Van Leeuwen and Gyorgyi [1923, 1], working with pure samples of lecithin and kephalin prepared by Levene showed that pure lecithin had no action on the hypodynamic heart of the frog, but that kephalin had a strong stimulant action, and that an ultrafiltrate of kephalin had only a slight stimulant action. They showed however [1923, 2] that the ultrafiltrate from kephalin produced as great an action as the original kephalin in sensitising the isolated gut of the rabbit to pilocarpin,

and that an action of similar intensity was produced by sodium oleate. The writers mention, moreover, that the kephalin they were using had undergone a certain amount of decomposition.

Pharmacological tests. My samples of lecithin and kephalin were tested upon the hypodynamic heart of the frog. All samples examined had been prepared recently and kept in nitrogen at 0°. The results of these tests are shown in the table and in Figs. 1 and 2.

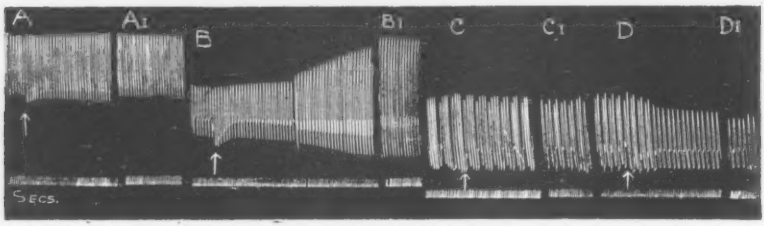

Fig. 1. Hypodynamic heart of frog (perfused 18 hours).
A. Perfusion with pure kephalin (0·002 %).
A 1. After 10 minutes' perfusion.
B. Perfusion with crude lecithin (egg) (0·002 %).
B 1. After 10 minutes' perfusion.
C. Perfusion with pure lecithin (egg) (0·004 %).
C 1. After 10 minutes' perfusion.
D. Perfusion with pure lecithin (brain) (0·004 %).
D 1. After 10 minutes' perfusion.

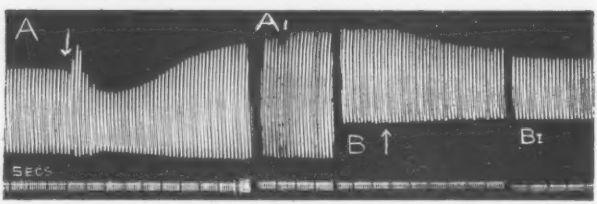

Fig. 2. Hypodynamic heart of frog (perfused 6 hours).
A. Perfused with water-soluble impurities extracted from lecithin (concentration less than 0·01 %).
A 1. After 10 minutes' perfusion.
B. Perfused with pure lecithin (brain) (0·01 %).
B 1. After 10 minutes' perfusion.

Pharmacological tests.

	Effect				
Concentration	Pure lecithin (brain)	Pure lecithin (egg)	Crude lecithin (egg)	Crude lecithin (brain)	Pure kephalin (brain)
1 : 25,000	Slight depression	Slight depression	400 % stim.	—	10 % stim.
1 : 50,000	—	—	400 % stim.	300 % stim.	Slight stim.
1 : 100,000	Doubtful effect	No action	400 % stim.	300 % stim.	Doubtful effect
1 : 200,000	No action	—	50 % stim.	—	—
1 : 500,000	—	—	No action	—	No action
1 : 1,000,000	—	—	—	—	—

At concentrations at which the crude lipin extracts of egg-yolk and ox-brain had powerful effects (10 parts per million) the pure phosphatides were without action. There would seem to be an impurity, perhaps a decomposition product (associated more particularly with kephalin), which is a strong cardiac stimulant. In this connection the activity of crude "lecithin" was compared with that of the water-soluble impurities extracted from it. Definite indications were obtained that the active principle was extracted by water (Fig. 2). Preliminary experiments showed that the water-soluble impurities also produced a stimulant effect on the guinea-pig's uterus but had no certain action on the isolated gut and uterus of the rat. The active principle or principles found cannot include choline, since this substance has a depressant action on the frog's heart. Sodium oleate may in part account for the action of the crude lipin extracts.

SUMMARY.

Samples of the phosphatides lecithin and kephalin have been prepared in a high degree of purity, particularly with respect to water-soluble impurities. In this state of purity neither phosphatide was found to be a cardiac stimulant, a result contrary to the experience of other workers. It is suggested that the activity of the specimens used by these workers was due to a water-soluble impurity, possibly of the nature of an organic base.

The author wishes to record his sincere thanks to Prof. A. J. Clark, who carried out the pharmacological tests and gave much valuable advice during the progress of the work and to Prof. J. C. Drummond for his constant help and encouragement. Thanks are also due to the Royal Society for a grant covering the expenses of the work.

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LIV. THE BIOLOGICAL SIGNIFICANCE OF THE UNSAPONIFIABLE MATTER OF OILS.

I. EXPERIMENTS WITH THE UNSATURATED HYDROCARBON, SQUALENE (SPINACENE).

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This paper is concerned with some feeding experiments on rats with squalene, a hydrocarbon of the formula $C_{30}H_{50}$ occurring in certain shark-liver oils, described by Tsujimoto [1916]. In a later paper [1920] he describes investigations carried out on 36 species of sharks, and it was found that the hydrocarbon occurred in the liver oils of numerous species and that these liver oils had in general a specific gravity of less than 0·9. Only in a very limited number of cases where the specific gravity of the oils was greater than 0·9 was squalene found and then in small quantity only.

Meanwhile Chapman [1917] independently isolated a hydrocarbon from the liver oils of *Centrophorus granulosus* and *Scymnorhinus lichia*; he gave it the name spinacene and attributed to it the formula $C_{30}H_{50}$. Later, however [1918, 1923], from a consideration of further results which had been obtained with larger quantities of material, he expressed the view that the formula of the substance should be $C_{29}H_{48}$ and not $C_{30}H_{50}$. Referring to Chapman's belief that the formula of spinacene was $C_{29}H_{48}$, Tsujimoto [1920] says: "Considering the fact that I have already prepared the hexahydrochloride of squalene of the formula $C_{30}H_{50}6HCl$, which has properties very similar to spinacene hexahydrochloride, $C_{29}H_{48}6HCl$, and also that spinacene has been obtained from an oil of the *Squalidae* sharks, it is highly probable that spinacene is identical with squalene." Further, in studies on the chemical nature of vitamin A, Drummond, Channon and Coward [1925] obtained very small quantities of the hexahydrochloride of the hydrocarbon from the unsaponifiable matter of cod-liver oil. Preparations of the hexahydrochloride were made from pure spinacene, and the highest value obtained for chlorine was 33·61 %, which agrees much better with $C_{30}H_{50}6HCl$ (Cl 33·86 %) than with $C_{29}H_{48}6HCl$ (Cl 34·63 %). These authors therefore also incline to the view that spinacene and squalene are identical and that the formula of the substance is $C_{30}H_{50}$. Other evidence points in the same direction; Prof. I. M. Heilbron, of the University of Liverpool, who has been working on this hydrocarbon and whose

results are about to be published [Heilbron and Kamm, 1926], has kindly consented to our making mention of such of his results as have a bearing on this work.

From a long and careful study of the hydrochloride of the hydrocarbon obtained from the liver oil of *Scymnorhinus lichia*, from which Tsujimoto isolated "squalene" ($C_{30}H_{50}$) and Chapman "spinacene" ($C_{29}H_{48}$), Heilbron and Kamm also arrive at the conclusion that squalene and spinacene are one and the same substance of the formula $C_{30}H_{50}$. As the bulk of the evidence points to the identity of these substances, and as "squalene" was described first [Tsujimoto, 1916], it is proposed to refer to the hydrocarbon in this paper as squalene and to adopt the formula $C_{30}H_{50}$.

Squalene is a colourless liquid hydrocarbon containing six double bonds. It forms a crystalline hexahydrochloride which is its most characteristic derivative. The melting point of this compound varies according to the method adopted in its recrystallisation (from acetone), but the usual preparation results in a substance melting at 113°. The crystals are hexagonal and diamond-shaped plates, but on rapidly cooling a solution in acetone fine needles may be obtained which appear to change into the plate form. The reasons for the variability of the melting point of the hydrochloride are given by Heilbron and Owens [1926]. This hydrochloride serves for the detection of the parent substance and reference has already been made [Drummond, Channon and Coward, 1925] to the fact that it can be obtained in variable yield by passing dry hydrochloric acid gas into a solution of squalene in a suitable solvent. Hitherto we have used ether and alcohol but a better yield can be obtained if acetone be used. Both these methods have been employed in the course of this work.

The occurrence of an unsaturated hydrocarbon in the liver oils of various fish is of peculiar biological interest. Tsujimoto [1920] gives a list of the 36 species of shark which he has examined; squalene is found chiefly in the *Squalidae*, but not every member of the order contains the hydrocarbon, which is also widely distributed among other Elasmobranchs. The amount of the hydrocarbon present in the liver oil varies very greatly. Usually fish-liver oils contain 1 to 2 % of unsaponifiable matter only, but the liver oils of some of the fish referred to may contain as much as 80 %, nearly all of which is squalene. It will be clear that this striking difference in the composition of the liver oil, which may contain only 20 % of true fat, must necessarily entail some abnormal metabolism which has not been hitherto described. Possibly squalene is formed as an intermediate compound which normally undergoes degradation into other products, and it may be that this process is for some reason or other hindered, with the resulting accumulation of the hydrocarbon. One important fact to be established is whether or not squalene is synthesised by the organism or is derived from the food ingested, and to this question we are turning attention. Further, the hydrocarbon appears not only in the livers of the fish, for Tsujimoto [1920] found it present in the egg oil of

Chlamydoselachus anguineus and *Lepidorhinus kinbei*. Heilbron and Kamm have also found squalene present in the eggs of *Etmopterus spinax*, *Lepidorhinus squamosus* and *Scymnorhinus lichia*. They examined 115 female specimens of *Etmopterus spinax* and in a number of them comparatively undeveloped eggs were found to contain squalene; in others in which development was almost complete, the yolk-sacs were found devoid of squalene, so that the substance was presumably absorbed during the course of development.

Reference is made in the next paper to the fact that there is a highly unsaturated hydrocarbon also present in the livers of man, ox, sheep, horse and pig. From results which have been so far obtained it would appear that this hydrocarbon is probably not squalene but a substance closely allied to it. The finding of a hydrocarbon of similar chemical nature in the mammal would suggest that this type of substance may be generally distributed, may possibly be of definite physiological importance in the metabolism of fats or allied substances, and that the occurrence of squalene in such quantities in fish livers may be a less remarkable phenomenon than would appear at first sight.

The work described in this paper was carried out with a view to determining whether squalene was absorbed by the rat; its occurrence in fish-liver oils suggested probable absorption, but the fact of its being a hydrocarbon of high molecular weight was against this view.

Experimental.

Experiment 1.

The general plan of the experiment was to feed two groups of rats on a complete artificial diet, while one group received in addition a small quantity of squalene each day. The diet used was that described by Drummond and Coward [1920], and was given to animals which had been so chosen that each group contained an animal of the same sex and weight. Immediately before feeding-time the animals were given approximately 0·05 cc. of squalene dropped directly into their mouths from a micro-burette. Records were taken of the amount of squalene given each day. The faeces were collected throughout the experiment at weekly intervals and stored in alcohol. Some little difficulty was encountered at first in finding the requisite amount of squalene to give to each rat per day, for beyond a certain daily dosage the substance exerts a marked purgative action which is in contrast with the behaviour of liquid paraffin which acts merely as a lubricant. The amount given was never in excess of 0·075 cc. per rat per day.

Treatment of livers. At the end of the experiment the animals were killed by chloroform and their livers removed as free from blood as possible. The combined livers of each group were weighed, heated with 5 % potassium hydroxide solution until solution was effected and extracted six or seven

times with ether in a separating funnel after adding sufficient alcohol to ensure rapid separation of the two layers. The combined ether extracts of each batch of livers were washed with water to remove soap and alcohol, evaporated to dryness and finally dried *in vacuo* at 100°. Excess of sodium ethylate in alcohol was then added and a second saponification carried out. Usually the residue from the ether extract after the first saponification consisted chiefly of unsaponifiable matter. After saponification with sodium ethylate, the solution was thrown into water and extracted with ether as before. Great care was taken at this stage to ensure by many washings with water that no soap was left behind in the ether extract. The ether extract was dried *in vacuo* at 100° and the weight of the unsaponifiable matter obtained. Cholesterol was quantitatively removed by the method of Windaus [1910], by adding an excess of a 1 % solution of digitonin in 90 % alcohol to a solution of the unsaponifiable matter in 95 % alcohol. After standing overnight the digitonide was filtered off, washed with ether and water to remove the excess of unsaponifiable matter and digitonin respectively, and weighed in a Gooch crucible. The mother liquor containing the unsaponifiable matter other than cholesterol was thrown into water, and exhaustively extracted with ether as before. After the usual washing of the ether extract, the ether was removed and the residue weighed after drying at 100° *in vacuo*; it was then dissolved in ether dried over sodium, and the solution saturated with hydrochloric acid gas. After standing overnight on ice the solution was filtered and the residue, if any, washed with ether to remove the adherent dark oily materials. The material so obtained was then recrystallised from hot acetone, filtered off and weighed. It was examined microscopically and its melting point determined.

The bodies of the animals were treated similarly as regards saponification. Cholesterol was, however, estimated in a small aliquot part. Squalene was not looked for in the unsaponifiable matter from the bodies.

Treatment of faeces. The faeces, which had been stored in alcohol, were ground up in the alcohol and filtered; two more grindings with absolute alcohol were made and the residue sucked as dry as possible. The residue was then extracted with ether for 3 days, re-ground and re-extracted for 1 day. All the alcohol washings and ether extracts were combined, and the solvents removed. Two saponifications followed by ether extractions were carried out as already described. Determinations of sterol were made by the modification of the method of Windaus [1910] introduced by Fraser and Gardner [1910]. Iodine values were also determined (Wijs). In this experiment the animals were killed 2 hours after receiving their last daily amount of squalene. The experiment lasted 42 days and each animal received 2·77 g. of squalene.

It will be more convenient to consider the results of all the experiments together later. It is to be pointed out, however, that as the experimental animals had received their daily dose of squalene just prior to being killed and as their alimentary tracts were not removed prior to the digestion of their bodies with potassium hydroxide, this amount of squalene (0·768 g.)

will appear in the weight of the unsaponifiable matter of their bodies. This figure will also include some part at least of the squalene given on the previous day. Hence it would appear desirable to subtract from the figure obtained (4·4474 g.), 1·536 g., as representing the amount of squalene administered on the day of killing and the preceding one. This gives a figure 2·9114 g. as the weight of the unsaponifiable matter of the bodies of the group of animals receiving squalene.

Exp. 1. Duration of experiment, 42 days.

	Control diet	Control diet + squalene
Number of animals	9	10·0
Squalene given, g.	0	27·7
Weight of livers, g.	64	78·0
Weight of bodies less livers, g.	1230	1405·0

	Livers		Bodies		Faeces	
	Control g.	Control + squalene g.	Control g.	Control + squalene g.	Control g.	Control + squalene g.
(*a*) Weight of unsaponifiable matter	0·3680	1·0774	1·8620	4·4474	2·6152	20·6890
Weight of cholesterol in (*a*)	0·2576	0·6189	1·3000	2·1620	1·2412	1·7150
(*b*) Weight of material not cholesterol in (*a*)	0·1004	0·4585	0·5620	2·2854	1·3742	18·9740
Weight of $C_{30}H_{50}6HCl$ obtained from (*b*)	0·00	0·14	—	—	—	—
Iodine value of (*a*) (Wijs)	—	—	135·4	177·1	80·4	316

It is clear from the weight of unsaponifiable matter from the faeces of the latter animals, and from its iodine value, that much of the squalene administered has been excreted. If we assume that that fraction of this unsaponifiable matter which is not squalene has an iodine value which is the same as that of the material from the control group, namely 80·4, then we may calculate from the iodine value of the whole of this unsaponifiable matter (316), that the amount of squalene present is 81·1 %, *i.e.* 16·79 g. (squalene has iodine value 371). This figure can only be approximate, but some rough check on it is obtained from the fact that, if we subtract the weight of material not sterol in the faeces of the control group from the corresponding figure for the faeces of the animals receiving squalene, we arrive at the figure 17·60 g. These figures 16·79 and 17·60 g. can only be approximate, but they would seem to show that about 17 g. of the 27·7 g. of squalene administered had been excreted. Thus about 10 g. have been absorbed during the course of 42 days and this absorption has resulted in a marked increase in the weight of unsaponifiable matter in the livers and also in the bodies of these animals, namely, from 0·3680 to 1·0774 g. in the case of the livers and from 1·8620 to 2·9114 g. in that of the bodies. There is also a large increase in the amounts of cholesterol, from 0·2576 to 0·6189 g. in the livers and from 1·3000 to 2·1620 g. in the bodies. The results are considered in more detail later.

Experiment 2.

The work described in Exp. 1 was repeated but the animals received 1 % of squalene in the basal diet instead of a fixed daily quantity by the mouth; they were killed 24 hours after their last meal of the squalene-containing diet. As the results of the first experiment had shown that some of the substance was absorbed, no estimation of squalene in the faeces was made because it is very difficult to keep such estimations quantitative when dealing with any bulk of material; and with a substance of such highly unsaturated nature the results so obtained lose some of their significance when regard is paid to the high proportion of squalene excreted and the probability of oxidation occurring. In this experiment and the remainder of the work, it was therefore decided to confine attention to the livers of the animals only; and these were treated as described under Exp. 1.

Exp. 2. Duration of experiment, 36 days. Animals killed 24 hours after receiving their last meal of the squalene-containing diet.

Number of animals	6
Weight of livers, g.	50
Weight of unsaponifiable matter, g.	0·5424
Weight of cholesterol, g.	0·3114
Weight of residue not cholesterol, g.	0·2310
Weight of $C_{30}H_{50}6HCl$ obtained, g.	0·09

Experiments 3 and 4.

As the results of the first experiments had indicated that squalene was absorbed and that it appeared in the liver, it became of interest to carry out experiments with a view to determining whether it disappeared from the liver after the cessation of the administration of the substance to the rats, and these two experiments were carried out with that end in view. The animals received the diet containing 1 % of squalene and were killed at varying intervals after the squalene-containing diet had been replaced by the basal diet only.

Exp. 3. Duration of experiment, 17 days on a diet containing 1 % squalene. Animals killed at various times after their last meal on this diet, receiving meanwhile the basal diet only.

Time, hours		Weight of livers g.	Weight of unsaponifiable matter g.	Weight of cholesterol g.	Weight of material not cholesterol g.
—	Control	42	0·1451	0·1079	0·0372
6	„ + squalene	54	0·5344	0·3062	0·2282
15	„ + „	49	0·4774	0·2434	0·2340
26	„ + „	48	0·5812	0·3378	0·2434
50	„ + „	36	0·4064	0·2448	0·1616
50	„	50	0·2056	0·1463	0·0593

There was isolated 0·11, 0·10, 0·11, 0·07 g. respectively of $C_{30}H_{50}6HCl$ from the animals receiving the squalene diet. The control materials gave none of this hydrochloride.

Exp. 4. Repetition of Exp. 3. Duration of experiment, 17 days.

Time, hours		Weight of livers g.	Weight of unsaponifiable matter g.	Weight of cholesterol g.	Weight of material not cholesterol g.
18	Control	35·4	0·1316	0·0923	0·0393
18	„ + squalene	29·9	0·3012	0·1747	0·1265
66	„ + „	38·3	0·4388	0·2681	0·1707
186	„ + „	38·0	0·2072	—	—

Discussion of Results.

As has been pointed out already, the first experiment shows that when squalene is administered to the rat, it is in part absorbed and as a result of this absorption there is a marked increase in the amounts of unsaponifiable matter and of cholesterol in the body and liver of the animal. Thus, even if the figure obtained for the weight of unsaponifiable matter in the bodies of the animals which received squalene be ignored because it is vitiated by the fact that they received their daily dose of squalene only a few hours before being killed, the big increase in cholesterol is significant. Thus, reducing the figures to the amount of cholesterol per kg. body-weight, we obtain for the control animals 1·058 g. and for those receiving squalene 1·539 g., an increase of about 50 %. A similar result holds for the livers in this experiment and, as the remainder of the work was carried out on the livers only, it will be convenient to consider all these results together.

The results are rendered clearer if a table be drawn up showing the weights of unsaponifiable matter, cholesterol and unsaponifiable matter not cholesterol, all the figures being calculated as grams per 100 g. of liver. The cholesterol is expressed as a percentage of the unsaponifiable matter.

		Livers (100 g.). Control diet				Control diet + squalene			
Exp.	Hours	Unsaponifiable matter g.	Cholesterol g.	Unsaponifiable matter not cholesterol g.	Cholesterol %	Unsaponifiable matter g.	Cholesterol g.	Unsaponifiable matter not cholesterol g.	Cholesterol %
1	3	0·5750	0·4025	0·1725	70·0	1·3830	0·7935	0·5895	57·4
2	24	0·4092	0·3069	0·1022	75·0	1·0848	0·6244	0·4604	57·5
3	6	0·3450	0·2697	0·0753	78·1	0·9895	0·5670	0·4225	57·3
	15	—	—	—	—	0·9743	0·4967	0·4776	52·0
	26	—	—	—	—	1·2108	0·7035	0·5073	58·1
	50	0·4122	0·2926	0·1186	71·2	1·1297	0·6800	0·4497	60·2
4	18	0·3717	—	—	—	1·0070	0·5843	0·4164	58·2
	66	—	—	—	—	1·1457	0·7000	0·4457	61·1
	186	—	—	—	—	0·6092	—	—	—
Mean		0·4226	0·3179	0·1171	73·6	1·1156	0·6437	0·4711	57·7

The amounts of squalene hexahydrochloride isolated from the livers of the animals which received squalene were 0·18, 0·18, 0·20, 0·20, 0·23 and 0·20 g. per 100 g. of liver in Exps. 1, 2 and 3, and 0·12 and 0·13 g. in the first two groups of Exp. 4. In calculating the mean values, the results obtained in the last group of Exp. 4 have been rejected as it would appear that the amount of unsaponifiable matter has reached a definitely lower figure and is not far removed from the average figure for animals fed on the basal diet only. The table shows that the unsaponifiable matter per 100 g. of liver of the animals

receiving squalene is 1·1156 g., as against 0·4226 g. in the livers of the control animals, an increase of 164 %. This increase is striking and coupled with it is an increase of just over 100 % in the amount of cholesterol, 0·6437 g. as against 0·3179 g., which results in there being a greater weight of cholesterol in the livers of the animals receiving squalene than there is total unsaponifiable matter in those of the control groups. It is to be noted that the increase in the non-cholesterol fraction of the unsaponifiable matter is not entirely accounted for by the presence in the livers of a corresponding amount of squalene, for although the hydrochloride of that substance was isolated from the livers, the amounts obtained were relatively small. Even allowing for the yield of this derivative being only 60 %, the actual amount of squalene present per 100 g. of liver would in no case exceed 0·2 g., whilst the average difference in the material not cholesterol is 0·3540 g.

It would be premature at this stage of the work to attempt to interpret this large increase of cholesterol in the livers of the animals receiving squalene with any certainty. Two possibilities arise—either squalene may be a precursor in cholesterol synthesis or else for some reason at present unknown the organ tends to adjust the ratio of cholesterol to other unsaponifiable matter present to as constant a value as possible. With regard to the first possibility, it is of interest to note that squalene, the molecule of which appears to consist of a long carbon chain containing six double bonds and branched methyl groups, yields a mixture of terpenes on distillation at atmospheric pressure, and Heilbron and Kamm [1926] comment on its possible relation to cholesterol. In this connection it is to be noted that although the present evidence points to it being normally absent from the liver, there seems to be present a substance which behaves in many ways like squalene [Channon and Marrian, 1926], and the fact that the animal body is capable of synthesising cholesterol [Channon, 1925] need not therefore negative the possibility of squalene being related to cholesterol.

The second suggestion as to the reason for the cholesterol increase would seem to receive some support from the fact that the increase in the unsaponifiable matter is not an increase in the cholesterol and squalene only, as already pointed out. It has been found in numerous experiments that the ratio of cholesterol to other unsaponifiable substances tends to a fixed value, as is shown for the figures obtained for the livers of the control animals in this experiment. The whole of these results may be due therefore to the absorption of squalene and its entry into the liver having caused an increase in cholesterol for some reasons of a physical nature. Further experiments are at present in progress to see whether an increase in the amount of the liver cholesterol can be caused by the administration of substances differing in chemical nature from squalene but having similar physical properties.

It will be seen from the results of Exps. 3 and 4 that there is no rapid return of the amount of unsaponifiable matter present in the liver to the normal value after depriving the animals of the squalene-containing diet, but the last

result obtained in Exp. 4 suggests that there is a distinct fall after a period of 8 days. In one experiment there seemed to be a sharp fall in the amount of unsaponifiable matter within 48 hours, but as all attempts to repeat this result have failed, it has been omitted from this paper. Further experiments are being carried out with a view to finding the rate of fall of the unsaponifiable matter to the normal value after the diet containing squalene is replaced by the basal diet.

No obvious ill effects followed the administration of squalene and *post mortem* examination showed that the organs of the animals were very healthy. There appeared to be a greater deposition of fat in the animals which received squalene and on the whole they seemed to grow at a somewhat greater rate than those of the control groups, possibly because the laxative action of the squalene caused a greater food consumption.

Summary.

1. When the unsaturated hydrocarbon squalene (spinacene) $C_{30}H_{50}$ is included in the diet of the rat, some absorption occurs.

2. This absorption results in the amount of unsaponifiable matter in the liver increasing to about 2·6 times the normal.

3. The amount of cholesterol present increases by about 100 %.

4. Squalene can be isolated, as the hydrochloride, from the livers of the animals which have received it in their diet.

5. The increase in the amount of unsaponifiable matter in the liver is not wholly accounted for by the presence of squalene and the increase in cholesterol.

6. The possible significance of these results is discussed.

7. No ill effects followed the administration of squalene to the rat.

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LV. THE BIOLOGICAL SIGNIFICANCE OF THE UNSAPONIFIABLE MATTER OF OILS.

II. AN UNIDENTIFIED UNSATURATED HYDROCARBON PRESENT IN MAMMALIAN LIVER.

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Reference has been made in the preceding paper [Channon, 1926] to work which is being carried out on squalene, a hydrocarbon of the formula $C_{30}H_{50}$, described by Tsujimoto [1916, 1920], which appears to be identical with a hydrocarbon, spinacene, isolated independently by Chapman [1917, 1918, 1923], who ascribed to it the formula $C_{29}H_{48}$. In that paper the biological interest of this highly unsaturated hydrocarbon, which is present to the extent of some 80 % of the liver oils of certain sharks, has been briefly discussed. Its occurrence outside the Elasmobranchs has been reported by Drummond, Channon and Coward [1925], who found it present as some 8 % of the unsaponifiable matter of cod-liver oil. Toyama [1923] reports the presence of another hydrocarbon, pristane, in fish-liver oils, and various alcohols of high molecular weight have been described, such as batyl alcohol, $C_{20}H_{42}O_3$, chimyl alcohol, $C_{19}H_{42}O_3$, and selachyl alcohol, $C_{20}H_{40}O_3$ [Tsujimoto, 1922, 1923, 1924]. The occurrence of substances such as these in fish-liver oils made it of interest to see whether they were present in livers other than those of fish, and this paper deals with an investigation into the unsaponifiable matter of the livers of certain animals which was undertaken with a view to proving the presence or otherwise of the unsaturated hydrocarbon, squalene, $C_{30}H_{50}$.

Preparation of the unsaponifiable matter from livers.

The preparation of this material was carried out by the method described in the previous paper. Briefly, the minced liver was heated in 5 % aqueous potassium hydroxide solution until it had dissolved, when the solution was cooled and strained into large separators where it was diluted with several volumes of water. The solution after the addition of alcohol was exhaustively extracted with ether and the combined ether extracts were washed with water

until free from soap, when the solvent was removed. The product so obtained was usually an oil containing much crystalline material (cholesterol) and was dried at 100° *in vacuo*. It was then resaponified with sodium ethylate to ensure the hydrolysis of esters of cholesterol and other higher alcohols possibly present and the solution, after being diluted many times with water, was extracted with ether as before. The ether extracts were then very thoroughly washed with water, and the ether evaporated. The final product was dried at 100° *in vacuo* and the flask sealed by a waxed cork. In this way were treated several kg. of human, pig, sheep, ox, and horse livers. These materials were stored for about 5 weeks with a view to their being worked up together. The livers, save in the first case, were taken from animals immediately after death, and were minced while still warm into potassium hydroxide solution on reception from the slaughter-house. The human material was some 48 hours old. The yields of unsaponifiable matter obtained were between 0·3 and 0·4 %. A similar figure was obtained for rats' livers [Channon, 1926], so that it would appear that in general there is present in liver some 3·5 g. of unsaponifiable matter per kg. wet weight. In the preparation, connective tissue was, as far as possible, excluded. The material so obtained varied somewhat in consistency but had as a rule a hard waxy appearance due to the presence of a large proportion of cholesterol, and was yellow to orange red in colour on account of the presence of the lipochrome pigments carotene and xanthophyll. That from man, ox, and sheep was very similar, deep red in colour and hard; that from horse liver was very much paler but of the same consistency, whilst material from the pig varied in colour considerably from very pale yellow to deep orange red, and appeared to be of a more oily nature although containing a very high proportion of cholesterol. No estimations were made of the cholesterol contents of these materials, but previous work has shown that there is usually present some 70 % of that substance.

Treatment of the unsaponifiable matter. In the previous paper mention has been made of the fact that squalene gives rise to a hexahydrochloride, $C_{30}H_{50}6HCl$, when hydrochloric acid gas is passed into its solution in ether or alcohol. This derivative, which crystallises in hexagonal and diâmond-shaped plates, is very characteristic and serves for the detection of the parent substance. Its melting point varies, but after two recrystallisations from acetone a white crystalline substance, M.P. 113°, results. It was decided to make use of the ready formation of this derivative in the search for squalene in these unsaponifiable liver materials. Some little difficulty was encountered at first owing to the large amount of cholesterol present, but a preparation of cholesterol hydrochloride showed that that substance crystallised in long silky needles, M.P. 155°, as stated by Mauthner [1906]. On recrystallisation from hot acetone it forms a gel which will not flow from an inverted tube. As it was hoped to recrystallise squalene hexahydrochloride from acetone, should that substance be found present, it was finally decided, after experiments on mixtures of cholesterol and squalene hydrochlorides, to overcome

this difficulty by removing the cholesterol derivative by grinding up the precipitate in a mortar with ether several times, and subsequently to recrystallise the ether-insoluble residue from acetone. The procedure adopted therefore was to pass dry hydrochloric acid gas into a solution of the unsaponifiable matter in ether and alcohol. The ratio of ether to alcohol was 4 : 1 and the concentration of unsaponifiable matter about 6 %. On passing hydrochloric acid gas into such a solution of the unsaponifiable matter, the colour rapidly changes to dark brown and finally goes quite black, and after about 12 hours a precipitate begins to appear. The solution after cooling on ice is filtered under reduced pressure when there remains a dark mass, amorphous for the most part, but containing also crystalline cholesterol hydrochloride. The latter is removed by grinding the mass in a mortar with ether and filtering and repeating this operation three or four times. The residue on drying is a light brown powder resembling in appearance the bromides of the highly unsaturated liver acids. This is dissolved in boiling acetone and filtered; there appear on cooling fawn-coloured crystals which under the microscope are seen to be very small needles which tend to form stellate clusters. Further recrystallisation does not result in any apparent improvement in colour, and we have been unable to obtain a white product. This substance did not seem to be the hydrochloride of squalene, for the latter can be readily obtained in beautiful white plates by crystallisation from acetone; it melted at 128° and contained about 31 % chlorine, whilst squalene hydrochloride melts at 113° and contains 33·86 % chlorine. The amounts of the substance we were obtaining at that time prevented any very definite evidence being obtained, but analysis made it appear that the substance which was giving rise to the hydrochloride was an unsaturated hydrocarbon other than squalene. This hydrochloride was originally obtained from the unsaponifiable matter of human liver; later an apparently similar substance of the same crystalline form, melting point, and chlorine content was obtained from the unsaponifiable matter of pig, ox, horse, and sheep liver. We thus tended to the belief that there was present in these materials a hydrocarbon which did not appear to be squalene; nevertheless the possibility that it was squalene which was giving rise to the hydrochloride was still kept in mind, for we could not be sure of the purity of such small amounts of material.

Formation of an insoluble bromide. Squalene gives rise to a bromide ($C_{30}H_{50}Br_{12}$) which can be formed by the addition of bromine in ether to an ether solution of squalene. The yield is by no means quantitative, and the reaction appears to be complicated by the formation of secondary products. The yield normally obtained is of the order of 35 %. It seemed reasonable to expect that the substance giving rise to the hydrochloride described above would also form an insoluble product on addition of bromine: this substance was readily obtained. On addition of excess of bromine to a solution of the unsaponifiable matter in ether, a precipitate is immediately formed, and, if this is filtered off, any cholesterol dibromide present in it can be readily

removed by frequent washings with ether. This substance, which is also not quite white, contains about 68 % bromine, is amorphous, and does not melt, but begins to char at 170° and goes black at 180°. Squalene dodecabromide behaves similarly when an attempt is made to melt it, but the latter contains 70·1 % bromine. In every case where a sample of unsaponifiable matter had given a hydrochloride by the method described above, it also gave a bromide on addition of bromine in ether.

It was therefore concluded that the unsaponifiable matter of the livers of man, ox, horse, sheep, and pig contains a hydrocarbon, which gives rise to a hydrochloride and a bromide very similar in halogen content to the corresponding squalene derivatives. The evidence suggested that this substance was not squalene, but no definite opinion was possible at that stage. It was therefore decided to work up a large quantity of material, and the liver of the pig was chosen, because there seemed to be present in the unsaponifiable matter of pig's liver a larger proportion of the unsaturated hydrocarbon than in the livers of the other animals used.

Preparation of unsaponifiable matter from 2 cwt. of pig's liver. Batches of ½ cwt. of pig's liver were treated in the manner described above for the preparation of the unsaponifiable matter. The yields obtained were not as quantitative as in the smaller preparations, owing to the difficulty of working up the material in bulk. The unsaponifiable matter obtained was stored in flasks completely filled with light petroleum. The livers used had come from animals which had been grass fed, and the unsaponifiable matter was an oily wax, dark red in colour. The four ½ cwt. batches of liver yielded 100, 94, 84, and 90 g. (mean 92 g.), which is 0·36 % of the wet weight of the liver.

Purification of the crude unsaponifiable matter. Squalene itself is miscible in all proportions with light petroleum, but its solubility in methyl alcohol is low (1·43 g. per 100 cc. at 15°). If the hydrocarbon present in these unsaponifiable matters bore any relation to squalene, therefore, its solubility in light petroleum and methyl alcohol should enable the bulk of the cholesterol to be removed, without appreciable loss of the hydrocarbon itself. This was investigated as follows: 1·3 g. of material from horse liver gave 0·113 g. of insoluble bromide; another 1·3 g. was boiled with 25 cc. methyl alcohol, cooled to room temperature and filtered; the filtrate after the usual treatment gave 0·041 g. of the bromide; the same quantity treated similarly with light petroleum instead of methyl alcohol gave 0·112 g. This experiment shows that extraction with light petroleum can be used for the removal of much of the cholesterol from the hydrocarbon without loss of the latter, and that the solubility of the substance in methyl alcohol at room temperature is very low.

The material, 368 g. in all, was therefore heated to boiling point with 1 litre of light petroleum and cooled. The mixture was shaken out on to a Buchner funnel and filtered; filtration was very slow and several changes of filter paper were necessary. The cholesterol remaining behind was washed

with light petroleum until it was free from the hydrocarbon. (Throughout this work we have used the formation of the insoluble bromide by the addition of bromine to an ether solution as indicating the presence of the hydrocarbon.) The cholesterol after drying *in vacuo* weighed 188 g. To ensure that the insoluble bromide formation was not due to the presence of traces of unsaturated fatty acids or to the presence of unhydrolysed fat from the original liver, the material contained in the light petroleum was resaponified with 25 g. of sodium dissolved in 350 cc. of alcohol in a current of nitrogen for 2 hours and for a further hour after the addition of 25 cc. of water. The usual ether-extraction was then carried out but considerable difficulty was encountered in washing the ether extract owing to the formation of emulsions. These emulsions have given trouble before when we have been dealing with ether solutions of squalene. The weight of material after this third saponification was 147 g. By bromination of 10 g. there was obtained 11·7 g. of bromide and, as the bromide contains 68 % bromine, the purity of the material giving rise to it must be 37·4 %, provided that one substance is giving rise to the bromide and that the yield is quantitative. The preparation of the hydrochloride from a known weight of material resulted in a yield corresponding to the original substance being 37·6 % pure. If we take the percentage purity therefore as 37·5, the 147 g. of the concentrated material must have contained 55 g. of the hydrocarbon; as this 55 g. originated from 2 cwt. of liver, the liver must have contained approximately 0·05 % of its wet weight as the hydrocarbon. It is to be noted that there is close agreement in the figures obtained for the purity of the material as given by the yields of hydrochloride and bromide. Further purification of this material, which was 37·5 % pure, was carried out by extraction with methyl alcohol. 5 g. were boiled with 150 cc. of methyl alcohol, cooled to room temperature and filtered. The insoluble material was a clear dark reddish-brown oil, and weighed 2·9 g. A determination of the weight of insoluble bromide obtained from a known weight of this 2·9 g., showed that it was now 63 % pure, for 0·29 g. yielded 0·57 g. of bromide. The substances extracted by methyl alcohol contained 27 % of cholesterol (digitonin method) and yielded only 0·3 g. of the bromide. The iodine value of the insoluble portion, 63 % pure, was 255 (Wijs); this fraction was again treated with 100 cc. of boiling methyl alcohol and the mixture cooled and filtered. The insoluble residue weighed 1·72 g., and 0·21 g. of it yielded 0·50 g. of the bromide corresponding to a purity of 76 %. Repetition of the methyl alcohol extraction, using 25 cc. of the solvent and filtering while the solution was hot, yielded 1·38 g. of the insoluble material and 0·07 g. of substance soluble in methyl alcohol. The former appeared to be 78 % pure by the method already adopted, and it was clear that purification by methyl alcohol extraction had been carried to its limit, for the last treatment had resulted in an increase of purity of only 2 %. This material therefore consisted of 78 % of the hydrocarbon and the remainder of other substances insoluble in methyl alcohol. It was a thick viscous oil, dark red in colour. Treatment of this

residue by boiling with charcoal in benzene solution resulted in a loss of material without any increase in purity.

The yield of squalene dodecabromide obtained by the addition of bromine to an ether solution of squalene varies from 22–45 % and the fact that the purity of this material as determined by the formation of the insoluble bromide was 78 % seems to be further evidence that the hydrocarbon is not squalene.

As methyl alcohol extraction had increased the purity of the substance from 37 to 78 %, it was decided to treat the main bulk in a similar manner. From the remaining 115 g. of thrice saponified material, a further 13 g. of cholesterol was removed by crystallisation from light petroleum. The remaining 102 g. was boiled with 250 cc. of methyl alcohol and filtered on cooling; the residue was again boiled with 250 cc. of methyl alcohol and filtered at 40°. The combined methyl alcohol mother liquors yielded 15 g. of crude cholesterol, and, after removal of this cholesterol, were again used for extracting the insoluble residue as before. After further treatment with 500 cc. and 250 cc. portions of fresh methyl alcohol, there remained behind 45 g. of material insoluble in methyl alcohol, whilst the methyl alcohol mother liquors contained 35 g. of material. The iodine value of the methyl alcohol-insoluble fraction was 309, and of the soluble portion 181. The former contained approximately 70 % of the hydrocarbon, and it was decided to submit it to distillation.

Distillation of the purified material. After a preliminary experiment on a small amount of material this was carried out on 27 g. at a pressure of 2 mm. There were obtained 1 cc. distilling from 200–225° and 1·5 cc. from 234–242°. These two fractions contained the cholesterol present in the material. The first fraction gave a very strong reaction with arsenious chloride, and was the usual vitamin A fraction [Drummond, Channon, Coward, 1925]. No further distillate was obtained until the temperature of the bath was about 350°. Distillation then recommenced and three fractions were obtained over the apparent temperature ranges of 264–290°, 292–296°, and 301–316°. Much decomposition, as evinced by white fumes, was apparent and the pressure rose to 8 mm. None of these fractions gave the usual insoluble bromide nor did the undistilled residue. Fractions 3, 4, and 5 appeared to be terpenes. Fraction 3 was a light mobile oil with a penetrating lemon-like smell; fraction 4 was more viscous and the last fraction still more so. This experiment shows that the hydrocarbon cannot be distilled at a pressure of 2 mm., but that if the temperature be raised sufficiently decomposition occurs and what appears to be a mixture of terpenes results. It was apparent that much of the distillate had been lost on account of lack of efficient condensation, for the material actually obtained was redistilled at 12 mm. with the following results:

	Temp. °C.	N_D^{20}	d_{20}^{20}
1.	65–100	1·4861	0·8672
2.	100–130	1·5020	0·8933
3.	130–200	1·5048	0·9020
4.	200–240	1·5092	0·9123
5.	240–270	1·5122	0·9222

Analysis of fractions 1, 2, and 4 gave the figures:

1. C, 85·2 %; H, 11·5 %; O (by difference), 3·3 %.
2. C, 86·2 %; H, 11·8 %; O ,, 2·0 % (calculated for C_5H_8: C, 88·23 %; H, 11·77 %).
3. C, 84·7 %; H, 11·5 %; O ,, 3·8 %.

These analyses show that the fractions contain oxygen, which is to be expected, for they were not analysed until some weeks after distillation and the readiness with which this type of compound takes up oxygen is well known. They would, however, seem to be of similar type to those obtained by Staudinger [1922] by the dry distillation of caoutchouc in a high vacuum and by Heilbron and Kamm [1926] by distillation of squalene at atmospheric pressure. It is of interest to note that squalene itself boils at 240° at 2 mm. and as the hydrocarbon with which we are dealing does not appear to distil at that pressure, it seems unlikely that it is squalene. The fact that the material which was used for the distillation was only 70 % pure prevents any rigid conclusion being drawn, but it would appear that the hydrocarbon may be closely related to squalene.

Analysis of the hydrochloride and bromide. The analytical figures for a number of preparations of these two derivatives are given in the following table. The hydrochloride was in each case recrystallised twice from acetone and washed with ether; it melted at 128°; the bromide was prepared as already described and ground with ether several times. Another preparation of the bromide (2 g.) was dissolved in 100 cc. of cold chloroform, and precipitated by the addition of 400 cc. of ether, and gave similar analytical figures. This bromide, like that of squalene, is insoluble in most solvents, but is somewhat soluble in pyridine, carbon disulphide, chloroform and tetrachloroethane; it does not melt, but darkens at 170° and goes black at 180°.

	Bromide			Hydrochloride		
	C	H	Br	C	H	Cl
	28·00 %	3·28 %	68·16 %	58·46 %	8·79 %	31·53 %
	28·33	4·08	67·76	58·81	8·81	30·83
	28·37	3·98	67·62	59·26	8·93	30·74
	28·50	3·99	67·90			
Mean	28·40	3·83	67·86	58·84	8·84	31·03

From these mean values it would appear that the bromide is pure; the figures for the hydrochloride show that that substance is only 98·71 % pure. We have been unable to obtain a purer product by repeated recrystallisation of the substance from hot acetone, for the crystals are contaminated by a brown oily material which washing with a large number of solvents has failed to remove. As to the nature of this impurity we have no information, but the hydrochloride, when first filtered after preparation, is black and although the bulk of this material can be removed by washing with ether and acetone, the small quantity remaining clings tenaciously to the hydrochloride and is not removed by recrystallisation. It would seem probable that this impurity is the result of partial decomposition of the hydrochloride itself. The microscopic appearance of the hydrochloride has already been mentioned. It is to

be pointed out, however, that the corresponding squalene derivative, which crystallises usually in hexagonal and diamond-shaped plates, does also give rise to needles very similar to those given by the hydrocarbon, but the former appears to be the more stable form. We have tried to obtain the hydrochloride of the new hydrocarbon in plate form similar to that of squalene hydrochloride and have failed repeatedly.

Unfortunately we have been able to determine the molecular weight neither of the hydrocarbon, on account of its impurity, nor of its derivatives, since neither the hydrochloride nor the bromide can be used cryoscopically on account of their low solubilities in any suitable solvents, whilst their tendency to decompose prevents use being made of the boiling point method or that based on the depression of the freezing point of camphor.

Some attempt has been made to regenerate the parent hydrocarbon from the hydrochloride and bromide. Squalene hydrochloride can be decomposed by boiling with sodium ethylate in alcoholic solution or by boiling with pyridine; it is even decomposed into its components by prolonged boiling in alcohol only [Heilbron and Kamm, 1926]. The hydrochloride has been submitted to the first two treatments with precautions taken to prevent oxidation, but, although the resultant oils have been highly unsaturated, they have failed to give solid products on the addition of bromine.

The chemical nature of the hydrocarbon.

It will be seen that very little evidence has been obtained as to the chemical nature of the hydrocarbon. The percentage composition of the hydrochloride and bromide are similar to those of the corresponding squalene derivatives, but with definitely lower halogen figures. In our opinion sufficient evidence has been adduced to enable us to say that the hydrocarbon is not squalene itself, and a number of facts suggest that it is possibly a higher homologue of that substance. Thus the hydrochloride is much less crystalline and its melting point is higher; the hydrocarbon does not distil at a pressure of 2 mm. but breaks down into a mixture of terpenes, whilst squalene itself distils at 240°/2 mm., but if distilled at atmospheric pressure, it also breaks down and seems to give rise to what appear to be very similar products [Heilbron and Kamm, 1926]. It is obvious that this evidence from the behaviour on distillation must not be regarded as being too definite, for the material distilled was only 70 % pure and the small amount of the products of distillation has prevented their study in any detail. There are given in the following table the percentage compositions required by derivatives of squalene and possible higher homologues and average figures obtained on those of the hydrocarbon.

	C	H	Br		C	H	Cl
$C_{30}H_{50}Br_{12}$	26·29 %	3·68 %	70·03 %	$C_{30}H_{56}Cl_6$	57·18 %	8·96 %	33·86 %
$C_{32}H_{54}Br_{12}$	27·47	3·86	68·67	$C_{32}H_{60}Cl_6$	58·42	9·20	32·34
$C_{33}H_{56}Br_{12}$	28·05	4·00	67·94	$C_{33}H_{62}Cl_6$	59·02	9·31	31·68
$C_{34}H_{58}Br_{12}$	28·62	4·10	67·28	$C_{34}H_{64}Cl_6$	59·55	9·40	31·02
X Br	28·40	3·83	67·86	*X* Cl	58·84	8·84	31·03

It will be seen from these figures that the addition of one carbon atom to the molecule does not entail a marked difference in the percentage figures, and as the amounts of the derivatives with which we have been dealing have been rather small, it would be wise not to lay too much stress on the results given by them, but to leave the question open until surer methods of purification have been devised. This preliminary note has been published because the preparation of a large amount of material will take some considerable time, and we are hoping to find a source of the hydrocarbon which will enable us to obtain it less contaminated with pigments and other substances having like solubilities. Hydrogenation may then result in the production of the corresponding saturated substance which may be distillable, since hydrogenated squalene, $C_{30}H_{62}$, distils at a lower temperature than squalene itself.

Note. Very large volumes of solvent have necessarily been used in the preparation of the material. Thus for each batch of ½ cwt. of liver some 20 l. of ether and 4 l. of absolute alcohol have been used. It was considered wise therefore to carry out a test on the reagents. Accordingly 10 l. of 5 % KOH solution were extracted with 10 l. of ether and 2 l. of alcohol and the extract treated in the same way as when dealing with the liver pulp. After drying at 100° *in vacuo*, there remained behind 0·11 g. of oil which did not give a solid bromine derivative. It was considered unnecessary to submit this to a second saponification. It has been our practice to use absolute alcohol, which has been treated with sodium and refluxed for many hours and then redistilled for the second saponification in order to minimise the amount of resinous material contaminating the final product. The residue from 5 l. of this alcohol to which had been added 200 g. of sodium over the course of several weeks and which was deep red in colour, was thrown into water and ether extracted in the usual way. It yielded 0·10 g. of a dark resin which, though unsaturated, did not give a solid bromine derivative. Another similar test carried out with a view to forming the hydrochloride also gave a negative result, as did a mixture of synthetic glycerides and cholesterol derivatives which was put through the complete process.

Summary.

1. There is present in the liver of man, ox, sheep, horse, and pig a highly unsaturated hydrocarbon which has not been isolated in a pure state.
2. The hydrocarbon yields a crystalline hydrochloride and a solid addition product with bromine. Analyses of these derivatives are given.
3. The hydrocarbon does not distil at a pressure of 2 mm., but decomposes, yielding what appear to be terpenes.
4. From a consideration of the results obtained it appears probable that the hydrocarbon is not squalene ($C_{30}H_{50}$), but a substance closely allied to it.
5. Squalene is apparently absent from the livers investigated.

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LVI. A MODIFICATION OF THE DEFLECTION BALANCE FOR USE IN BIOCHEMICAL LABORATORIES.

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The instrument to be described was evolved for the purpose of weighing out small quantities of reagents of the order of 10 mg. more rapidly than is possible on an ordinary chemical balance. It has proved very easy to use and may find other uses. It can replace the torsion balance of any range and costs approximately £2. 10*s*. 0*d*. to make. Similar deflection balances have been used before [Salvioli, 1919; Research staff of G.E.C., 1923]. Salvioli used glass fibres, which are too fragile for common use, and observed the deflection with a microscope. The G.E.C. instrument has a steel wire but no scale pan and the deflection is observed by projection. The microscope (or projection system) has been replaced in the instrument to be described by a simple magnifying glass and the glass fibre by a steel wire which can be quite roughly handled without altering its calibration value.

The base of the balance consists of a wooden block *A* mounted on tripod screws *P*. Fastened to the top of this is a brass plate *L*, which, to ensure rigidity, is attached by eight screws. On top of this at one end is attached the brass or wooden block *J*, and fastened to the top of this is a brass or steel block *K'* with a longitudinal V-shaped groove in its upper surface. *K* is another similar block, with a groove on its lower surface, which is held on by the knurled nuts *M*. A small brass rod *R* is held between *K* and *K'*, and to the end of this rod is soldered a tinned steel wire *B*, 0·039 mm. in diameter. The other end of this wire is bent downwards as in the figure and is rounded off on a hone. The deflection of this wire is used as the index of the weight of material placed in the scale pan *E*, the position of which is fixed in relation to the wire by bending the wire *B* into the hook shown in the figure. The scale pan (see Fig. 1 *d*) is made of a thin piece of mica *F*, square in shape, to which is attached, by means of Chatterton compound, the fine (40 gauge) copper wire *G* bent into a hook which rests on the hook on the wire *B*. The weight of the pan is about 18 mg. On one occasion, a very dry frosty day, the pan and the slip of mica carrying the powder to be weighed were found to become electrified by friction, and to be difficult, for that reason, to manipulate. This difficulty was overcome by gently breathing on the mica in

order to render the surface an electrical conductor[1], or the pan may be covered with a thin deposit of platinum by immersing it in 1 % H_2PtCl_6 in bergamot oil and heating slowly to just below redness. The wire and pan are covered with a glass hood in a metal frame *I* hinged at *C*.

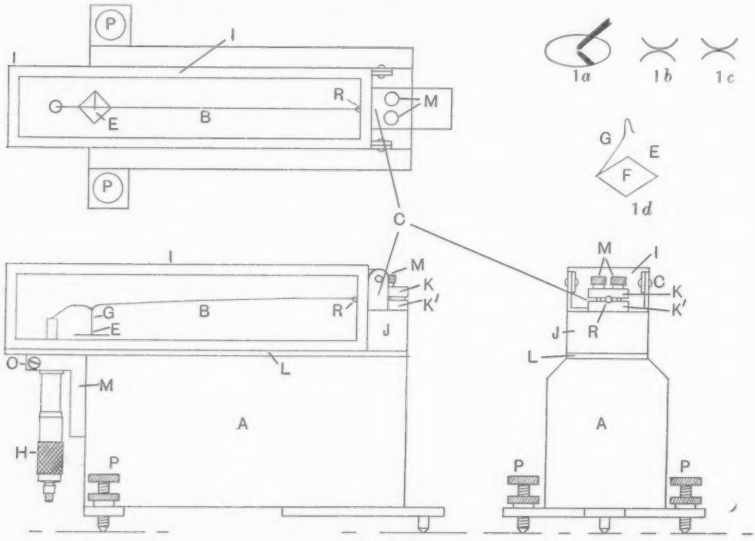

Fig. 1.

The object to be weighed is placed on this pan, powders being weighed on a separate piece of mica placed on the pan. The excess of weight causes a deflection of the wire *B*, which is measured by the micrometer head *H* mounted in the angle block *N* and passing through a hole in the brass plate *L* (Starrett micrometer head obtainable at engineers' tool-shops). The zero reading is first obtained with the pan empty. The micrometer is then screwed up until the end of the micrometer plunger just touches the wire, contact being observed through a lens magnifying seven times. The actual point of contact can be decided with great accuracy because there is a reflected image of the end of the wire in the upper surface of the micrometer plunger (Fig. 1 *a*). The wire is rounded off and rests tangentially on the plunger as in the diagram. If a lamp is placed behind and a little to the left of the micrometer the disappearance of the fine line of light between the image and the wire enables the position of the wire to be determined with an accuracy of 0·001 mm. Just before contact occurs the wire and its image are separated by a fine line of light as in Fig. 1 *b*. At the moment of contact the fine white line is apparently replaced by a fairly broad surface of contact the shape of which is represented

[1] The procedure described for the removal of an electrical charge by breathing on the pan is not so unsound as it might appear. The amount of moisture condensing on the pan is quite beyond the range of detection by weighing although sufficient to dissipate the charge.

in Fig. 1 *c*. This method of measuring small lengths is, of course, not new, but is generally used with a pool of mercury as a mirror. The setting up of the wire so as to give a deflection of any convenient value is carried out in the following manner.

A wire is chosen of about the dimensions required and one end bent down and rounded off. A hole is drilled along the long axis of a piece of brass rod *R*, 1 inch long by 3/16 inch diameter, of such a size as to be a sliding fit for the straight end of the wire. The rod is then clamped between the flat pieces of steel *K* and *K'* by knurled nuts *M* and a point on the wire found where the application of a 10 mg. rider deflects the bent end of the wire 1 millimetre. A hook is then made as nearly as possible at this point and the wire slid in or out of the brass rod *R* until the deflection produced by 10 mg. is exactly 1 mm. again. The wire is then fixed to the rod by solder. The degree of accuracy obtainable is illustrated by the tables. These represent an average of 3–5 readings at each point, and the worst agreement amongst the series of readings is shown below, Table II.

Table I.

Total load in pan (weight of pan 18 mg.)	Deflection: mm. for 10 mg.
10 mg.	1·00125
30 „	1·006
50 „	0·9977

The same 10 mg. rider was added (column 2) at each stage, other weights (column 1) being made up from a box of weights, and the readings in the second column represent the increment at each load produced by the addition of this rider.

Table II.

Total load on pan	Deflection in mm. on addition of a reputed 1 mg. weight
1	0·101
11	0·1014
16	0·1015
31	0·1007
41	0·104

This "1 mg." weight is obviously rather more than $\frac{1}{5}$ of the weight of the 10 mg. rider used for the first table. The worst agreement obtained amongst readings of any of the above points was at a load of 40 mg. and the readings were:

8·582, 8·583, 8·585, 8·584, 8·585 mm.

In weighing, the substance is placed either directly on the pan or on a thin flake of mica which is placed on the pan. It will be seen that up to a total load of between 30 and 40 mg. the readings are proportional to the weight with an accuracy of $\pm$ 0·01 mg. The calibration can be made to depend on one 10 mg. rider and can be checked in a few seconds at any time. The deflection for 10 mg. has remained constant for 8 months, and readings for the same rider never differ by more than 0·01 mg.

The apparatus is covered with a glass hood *I* to keep off draughts and dust, the reading being made through the glass. The top of the micrometer

and the wire need dusting with a camel hair brush occasionally and should be gently polished with metal polish to prevent sticking. A series of wires of different thicknesses of ranges 0·1 to 1, 1 to 10, 10 to 100, and 100 to 1000 mg. can be made and used in the same instrument.

SUMMARY.

A cheap and simple form of balance is described, the action of which depends on the bending of a steel wire. By its means 1 to 30 mg. can be weighed to ± 0·01 mg. in 30 seconds. By using a series of wires of different thicknesses on the same instrument, the replacement of the wire by another taking only a few seconds, any range of weights from 1 mg. to 1 g. can be weighed with an accuracy of ± 1 in 10,000.

I take this opportunity of thanking my mechanic, F. Gowlett, for his assistance.

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LVII. THE ESTIMATION OF CALCIUM IN BLOOD-SERUM.

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(*Received March 11th, 1926.*)

The method for the estimation of calcium in blood-serum devised by Kramer and Tisdall [1921] has been extensively used and, it is generally agreed, gives reproducible results in expert hands. Clark and Collip [1925], however, emphasise that the details of the method must be followed with meticulous accuracy or discrepant results will be obtained. They attribute the main source of error to the slight solubility of calcium oxalate and are of opinion that traces of ammonium oxalate, used for washing the precipitate, remain, and, if close adherence to the original method is maintained, tend to compensate for the error due to solution of the calcium oxalate. In consequence too much washing, or too little, will give inaccurate results. The original method involves titration of the precipitated oxalate with $N/100$ potassium permanganate. One of us, apart from an instinctive aversion from permanganate titrations, finds it extremely difficult to decide when the end point is reached although the other author can, by the exercise of some patience, obtain reproducible titration figures. It occurred to us that by the conversion of the oxalate into carbonate the use of permanganate could be avoided, whilst the amount of carbonate could be estimated by acidimetric methods.

Hamilton [1925] has used a similar modification of the Kramer and Tisdall method which was published before the working out of our method was completed, but the details of the method we use make a considerable difference in the time necessary to complete an estimation, as compared with that required by the method used by Hamilton.

The method also has the advantage that the calcium oxalate need not be washed with water at any stage, saturated ammonium oxalate in which calcium oxalate is absolutely insoluble, being used instead. Any excess of ammonium oxalate is decomposed and driven off as carbonate when the final heating stage is reached.

Method.

Apparatus.

(1) Small centrifuge tubes of the dimensions shown in the figure and made of resistance glass (*e.g.* Duroglass). We do not find that quartz tubes are necessary. The shape of the bottom of the tube is of importance; the sides of the conical end must be sufficiently steep for the oxalate precipitate to slide down to the apex of the cone during centrifuging. If any oxalate adheres to the sides of the tube there is a chance of losing some during decantation of supernatant liquid. The bottom of the tube inside should be just rounded off.

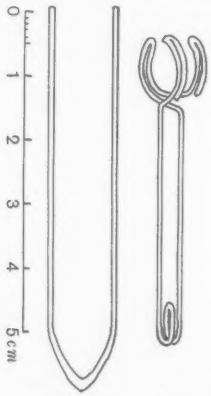

Fig. 1. Centrifuge tube and wire holder.

(2) 1 cc. pipettes.

(3) Glass stirring rods 10 cm. by 1·5 mm. made from drawn-out tubing.

(4) Some form of micro-burette. The final titration for 1 cc. of serum consists of the addition of about 0·25 cc. of *N*/50 alkali. For this purpose we use the micrometer syringe described by one of us [Trevan, 1925]. A Rehberg burette would serve probably as well; or by using 2 cc. of serum as in the original Kramer and Tisdall method, together with *N*/100 alkali for the final titration, an ordinary 2 cc. burette could be used. In this case, however, the end-point error would be rather larger.

(5) Centrifuge.

(6) Wire holder for tube (see Fig. 1).

Reagents.

(1) Saturated ammonium oxalate (3·5 %).

(2) *N*/50 sodium hydroxide.

(3) *N*/100 acid. We have used both phosphoric acid, as recommended by Cahen and Hurtley [1916], and hydrochloric. We prefer the former. The acid should be titrated against *N*/50 Na_2CO_3 by a special method; excess of acid should be added to the standard carbonate and the amount un-neutralised titrated with the *N*/50 sodium hydroxide. The conditions of the calcium carbonate titration are thus more closely reproduced and the titration error diminished.

(4) Indicator. We have found bromophenol blue (0·04 %) the best of several we have tried. Methyl red (0·02 %) is almost as good but has to be used near the acid end of its range, and turns rather too far on the alkaline side to be entirely satisfactory for H_3PO_4. Buffer solutions, p_H 4·0 and 4·2 for bromophenol blue, 4·4 and 4·6 for methyl red, are used as comparison solutions for judging the end point. The titration of the $N/100$ acid against standard carbonate should of course be carried out against the same indicator as that finally used for the titration of the calcium carbonate.

Procedure.

2 cc. of ammonium oxalate are measured into one of the centrifuge tubes and 1 cc. of serum is added. The operation is best carried out in this order, as, if the serum is measured into the tube, the first drops may dry on the side of the tube before the oxalate is added and prove difficult to re-dissolve afterwards. The contents of the tube are then stirred vigorously with one of the small glass stirring rods which is then withdrawn. The amount of fluid adhering to the stirring rod we find to be of the order of 0·3 mg. and may therefore be neglected. The tubes are allowed to stand for 2–3 hours and are centrifuged at about 3000 revolutions per minute for 10 minutes. The supernatant fluid is poured off and the tube drained by inversion over clean filter paper as recommended by Clark and Collip. After draining for some minutes 2 cc. of ammonium oxalate are added and the tube centrifuged again. The oxalate is removed, a fresh amount added, and the centrifugation once more repeated. At each centrifugation the oxalate should pack in the very apex of the conical end of the tube if the taper is correct. The tubes are then dried in a steam oven to prevent spurting in the next stage. The conversion into carbonate is carried out by holding the tube in the wire clip shown and passing it through the Bunsen flame for one minute. The ammonium oxalate left is converted into ammonium carbonate which comes off as a white cloud. Care should be taken to heat the whole of the tube to drive off any ammonium carbonate that may condense on the cooler parts. The temperature required for complete conversion of the carbonate is comparatively low. Some rough experiments carried out in a muffle show that it is not higher than 250°. Overheating is to be avoided as the calcium oxide formed may prove very difficult to dissolve in $N/100$ acid. The correct temperature, which is not difficult to arrive at, is indicated by the first appearance of the sodium flame around the tube. The tube is cooled, 1 cc. of $N/100$ acid added and allowed to stand for some minutes. We find that, as Cahen and Hurtley claim, the rate of solution is greater in H_3PO_4 than in HCl and the solution may be gently warmed if H_3PO_4 is used. When the solution of the carbonate is complete one drop of 0·04 % bromophenol blue is added from a very fine capillary pipette (the volume of the drop we use is 0·016 cc.). The titration is carried out with the micrometer syringe held vertically in a burette clamp. The addition of the sodium hydroxide is carried out by running a drop on to one of the small

stirring rods and then transferring it to the solution. The smallest drop which can be dealt with in this way has a volume of about 0·00015 cc., which is more than sufficiently small for accurate titration. This corresponds to about half a division on a metric micrometer head for the average "tuberculin syringe." The formation of drops of this size and their adequate removal by the stirring rod are much facilitated by coating the exterior of the needle of the syringe with paraffin wax, as recommended by Wright and Colebrook [1921]. The needle may be either the finest steel hypodermic needle or the glass needle described with the micrometer syringe. The latter is preferable as it is easier to see that it is filled to the tip when the titration is begun. Titration is carried to the point where the solution has a colour intermediate between those of buffers of p_H 4·0 and 4·2 each with a concentration of bromophenol blue equal to that of the solution which is being titrated. The difference between the titration figure so obtained and the titration figure for the acid alone gives the amount of calcium in the serum taken.

Results.

Solution of calcium chloride containing 0·091 mg. per cc. made by dissolving calcium carbonate in hydrochloric acid:

0·09140, 0·0918, 0·0916, 0·0914 mg. Ca per cc.

Horse serum: 8·6, 8·54 mg. Ca per 100 cc. on the same sample.

Rabbit serum: 13·26, 13·20, 13·16 mg. Ca per 100 cc.

We have obtained figures within less than 5 % of the correct value when using the same method with 0·01 mg. Ca per cc., measuring all solutions with a micrometer syringe and keeping the tubes covered during standing and centrifugation with rubber caps, such as are used for vaccine bottles. We have, however, not often used these small quantities.

Summary.

A method of estimating calcium is described in which the calcium is precipitated as oxalate and converted into calcium carbonate by heating the centrifuge tube in which it is separated and washed. The calcium carbonate is titrated with acid.

The method has the advantages that the end point is more distinct than that of a permanganate titration, and all washing is carried out with saturated ammonium oxalate, thus avoiding errors due to the solubility of calcium oxalate in water.

By the use of the micrometer syringe 1 cc. only of serum is required and smaller quantities down to 0·1 cc. will give results accurate enough for most purposes.

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LVIII. THE INFLUENCE OF THE FATTY ACIDS AND THEIR SALTS ON ALCOHOLIC FERMENTATION BY LIVING YEAST.

PART I. ACETIC AND FORMIC ACIDS AND THEIR SODIUM, POTASSIUM AND AMMONIUM SALTS.

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(*Received March 8th, 1926.*)

In order to ascertain what effect a given substance has on the fermentation of sugar by living yeast, it is necessary in the first place to decide on an appropriate standard method of determining the normal rate of fermentation. When a sugar solution is treated with yeast the liquid gradually increases in acidity and a variable rate is observed. The use of a buffer is therefore essential and the question then arises how variation in the concentration and composition of the buffer affects the rate of fermentation. The following experiments were instituted in order to obtain some satisfactory basis for the comparison of the action of various salts, etc., which have from time to time been stated to have an accelerating or depressing effect on the rate of fermentation by living yeast.

The relation between rate of fermentation and p_H value has been a matter of research for many years. Many authors have already discussed the question whether the rate of fermentation reveals any remarkable difference between the various acids employed to regulate the p_H value of the medium. Hägglund [1914] tested Bial's conclusions [1902] as to various acids, and pointed out the relation between the influence of an acid upon the rate of fermentation and the degree of dissociation of the acid. On the other hand, Euler and Heintze [1919] observed that weak acids such as oxalic, chromic and acetic acids have a marked specific poisoning effect on yeast above a certain concentration.

Recently Hägglund and Augustson [1925, 1, 2] have worked with buffer solutions composed of acetic acid-acetate, lactic acid-lactate and phosphoric acid-phosphate, and observed that the optimum p_H value depends upon the variety of sugar, period of observation and kind of acid. They also noted that yeast is very sensitive to free acetic acid.

In the present experiments the effects of the salts of acetic and formic acids on the rate of fermentation by living yeast have been studied with the special object of ascertaining the influence of concentration of hydrogen ions, and the nature and concentration of the free acids and their salts.

Methods.

The apparatus described by Harden, Thompson and Young [1910] was used. 20 cc. of 10 % glucose solution and 20 cc. of the various buffer solutions were placed in a 50 cc. flask, saturated with CO_2, connected with the mercury burette, and incubated in a water-bath at 25°. 10 cc. of the solution was pipetted out, and its p_H value was colorimetrically observed by the capillator method. 2 g. of pressed top yeast were added to the remaining 30 cc. of solution and the amount of evolved CO_2 was observed at intervals of 5 minutes for nearly 1 hour. The liquid was then filtered, the filtrate saturated with CO_2, incubated, and the p_H value again determined by the above method. For the rate of fermentation, the average number of cc. of CO_2 evolved in 5 minutes was chosen. In order to allow for variation in the fermenting power of different samples of yeast, a fermentation was always carried out in presence of a 0·2 *M* acetate solution at p_H 4·7, which was made by mixing two volumes of 0·2 *M* sodium acetate and one volume of 0·2 *M* acetic acid. The standard rate of fermentation under these circumstances was taken as 2·4 cc. per 5 minutes. When the observed rate differed from this, the ratio of the two numbers was found and used as a factor to correct all the observations of the series to the standard.

In order to test the validity of this method a series of experiments was made in which a number of different samples of yeast, or the same sample after being kept for various periods, were allowed to ferment the same series of solutions.

Exp. 1. Four solutions were used, made by mixing 0·2 *M* potassium acetate and 0·2 *M* acetic acid, and compared with the standard made up as described above with two parts of 0·2 *M* sodium acetate and one part of 0·2 *M* acetic acid. The concentration in every case is therefore 0·2 *M* and the solutions were as follows:

No. of solution	Ratio of acetate/acetic acid	Mean rate of fermentation cc. per 5′	Same yeast after keeping		
			For 24 hours at 0°	For 2 days at 0°	For 3 days at 0°
1 K acetate	2 : 5	0·02	0·17	0·2	0·12
2 „	2 : 1	2·6	2·9	2·6	2·65
3 „	5 : 1	6·1	6·25	6·5	6·4
4 „	1 : 0	8·24	8·25	7·6	7·8
5 Na acetate	2 : 1	1·76	1·87	1·86	1·78

The p_H values did not alter during the fermentation.

Exp. 2. In this case a different sample of yeast was used with the same set of solutions.

	Number of solution				
	1	2	3	4	5
I. Fresh yeast	0·05	1·75	4·6	6·0	1·1
II. After keeping for 1 day	0·1	1·97	4·5	6·2	1·4
III. „ „ „ 2 days	0·14	1·8	4·0	5·7	1·3
IV. „ „ „ 3 „	0·16	1·6	3·9	5·5	1·2

Taking the values for solution No. 5 as standard and making the rate in each case equal to 1 we get the following relative values by dividing the value for each solution by that of the standard (No. 5) in each series.

	Exp. 1. Number of solutions					*Exp.* 2. Number of solutions				
Series	1	2	3	4	5	1	2	3	4	5
I	0·01	1·48	3·47	4·68	1	0·04	1·56	4·04	5·33	1
II	0·09	1·55	3·34	4·41	1	0·07	1·42	3·27	4·44	1
III	0·11	1·40	3·36	4·09	1	0·11	1·36	4·05	4·27	1
IV	0·07	1·49	3·52	4·38	1	0·13	1·33	3·19	4·49	1
Average	0·07	1·48	3·42	4·39	1	0·09	1·42	3·64	4·63	1

It will be seen that the values in the various series and the average values for the two different samples of yeast agree very well, although the actual rates observed with the second sample of yeast were considerably lower than with the first.

Concentration of free acid.

The concentration of free acid in the medium is equal to that added + the negligibly small amount formed by hydrolysis of the salt + the amount of acid produced from the salt in the process of saturation with CO_2. In order to ascertain the last amount, the following experiments were carried out.

To 30 cc. of mixtures having the same composition as the solutions used in fermentation, 10 cc. of HCl of suitable strength was added after saturation with CO_2, and the evolution of CO_2 was measured in the same apparatus as was used for the fermentations. The results have not been corrected for any possible error due to the altered solubility of CO_2 in the solutions after mixing, but this is not likely to be appreciable. From the amount of CO_2 thus obtained the total quantity of free acid can be easily calculated by using the equation:

$$CH_3COONa + H_2CO_3 = CH_3COOH + NaHCO_3.$$

A single example is given below of the case of sodium acetate to which no free acetic acid has been added. The results obtained are incorporated in Table I.

Concentration of Na acetate (*M*)	0·5	0·2	0·1	0·05	0·025
Concentration of HCl (*M*) added	2·0	0·8	0·4	0·2	0·1
cc. of CO_2 evolved (N.T.P.)	12·19	5·56	4·39	2·6	1·79
Concentration of free acetic acid (*M*)	0·018	0·008	0·006	0·004	0·003

Results.

Table I gives the results of the experiments.

Discussion.

The curves (Fig. 1) when the rate of fermentation is plotted against the p_H are very similar for equal concentrations of the buffer solutions composed of acetic acid and Na acetate, K acetate and NH_4 acetate respectively. With these acetate-acetic acid buffer solutions, the rate of fermentation at constant

Table I.

A. *Sodium acetate and acetic acid.*

Ratio (Salt/Acid)	Concentration of total acetate (*M*)	Concentration of $C_2H_4O_2$ (*M*)	Corrected rate of fermentation cc. per 5′	*p*H Beginning	*p*H End
Exp. 1.					
2 : 5	0·5	0·357	0·04	4·1	4·1
2 : 1	0·5	0·167	0·7	4·7	4·7
5 : 1	0·5	0·085	2·5	5·1	5·1
1 : 0	0·5	0·018	5·7	6·2	6·2
2 : 1 (standard)	0·2	—	2·4	4·7	4·7
Exp. 2.					
2 : 5	0·2	0·143	0·2	4·0	4·0
2 : 1	0·2	0·067	2·4	4·7	4·7
5 : 1	0·2	0·034	7·3	5·1	5·1
1 : 0	0·2	0·008	9·1	6·0	5·9
Exp. 3.					
2 : 5	0·1	0·071	1·4	4·0	4·0
2 : 1	0·1	0·033	6·5	4·7	4·7
5 : 1	0·1	0·018	8·8	5·0	5·0
1 : 0	0·1	0·006	10·8	5·7	5·4
Exp. 4.					
2 : 5	0·05	0·036	4·8	4·0	4·0
2 : 1	0·05	0·017	8·1	4·7	4·6
5 : 1	0·05	0·009	10·5	5·0	4·8
1 : 0	0·05	0·004	11·6	5·4	5·0
Exp. 5.					
2 : 5	0·025	0·018	7·0	4·0	3·8
2 : 1	0·025	0·008	9·3	4·6	4·3
5 : 1	0·025	0·005	10·1	4·9	4·5
1 : 0	0·025	0·003	11·4	5·4	4·7

B. *Potassium acetate and acetic acid.*

Ratio (Salt/Acid)	Concentration of total acetate (*M*)	Concentration of $C_2H_4O_2$ (*M*)	Corrected rate of fermentation cc. per 5′	*p*H Beginning	*p*H End
Exp. 6.					
2 : 5	0·5	0·357	0·01	4·2	4·2
2 : 1	0·5	0·167	0·9	5·0	5·0
5 : 1	0·5	0·085	3·1	5·3	5·3
1 : 0	0·5	0·025	6·0	6·2	6·2
Exp. 7.					
2 : 5	0·2	0·143	0·3	4·2	4·2
2 : 1	0·2	0·067	2·3	4·9	4·9
5 : 1	0·2	0·035	5·0	5·3	5·3
1 : 0	0·2	0·013	8·6	6·1	5·8
Exp. 8.					
2 : 5	0·1	0·071	2·3	4·1	4·1
2 : 1	0·1	0·033	7·1	4·8	4·8
5 : 1	0·1	0·018	9·0	5·2	5·2
1 : 0	0·1	0·007	10·2	5·8	5·6
Exp. 9.					
2 : 5	0·05	0·036	5·7	4·1	4·0
2 : 1	0·05	0·017	8·4	4·8	4·6
5 : 1	0·05	0·009	9·8	5·2	4·9
1 : 0	0·05	0·005	10·4	5·6	5·2
Exp. 10.					
2 : 5	0·025	0·018	7·5	4·1	3·7
2 : 1	0·025	0·008	9·6	4·7	4·5
5 : 1	0·025	0·005	10·1	5·0	4·7
1 : 0	0·025	0·002	10·1	5·4	4·9

C. *Ammonium acetate and acetic acid.*

Exp. 11.					
2 : 5	0·5	0·357	0·02	4·0	4·0
2 : 1	0·5	0·167	0·2	4·7	4·7
5 : 1	0·5	0·084	1·1	4·9	4·9
1 : 0	0·5	0·013	6·9	6·2	6·1
Exp. 12.					
2 : 5	0·2	0·143	0·2	4·0	4·0
2 : 1	0·2	0·067	1·6	4·6	4·6
5 : 1	0·2	0·033	3·6	4·9	4·9
1 : 0	0·2	0·007	10·0	5·9	5·7
Exp. 13.					
2 : 5	0·1	0·071	2·0	3·9	3·9
2 : 1	0·1	0·033	6·2	4·6	4·6
5 : 1	0·1	0·017	9·7	5·0	4·9
1 : 0	0·1	0·006	11·7	5·5	5·2
Exp. 14.					
2 : 5	0·05	0·036	5·5	4·0	3·9
2 : 1	0·05	0·017	9·9	4·6	4·4
5 : 1	0·05	0·008	11·1	5·0	4·7
1 : 0	0·05	0·003	11·8	5·4	5·0
Exp. 15.					
2 : 5	0·025	0·018	8·1	3·9	3·8
2 : 1	0·025	0·008	10·6	4·6	4·1
5 : 1	0·025	0·004	11·5	4·9	4·3
1 : 0	0·025	0·001	11·8	5·3	4·5

D. *Sodium formate and formic acid.*

Ratio (Salt/Acid)	Concentration of total formate (*M*)	Concentration of CH_2O_2 (*M*)			
Exp. 16.					
5 : 1	0·5	0·083	0	4·1	4·2
15 : 1	0·5	0·031	0·03	4·6	4·6
1 : 0	0·5	0·004	5·9	5·7	5·4
Exp. 17.					
5 : 1	0·2	0·033	0·04	4·1	4·2
15 : 1	0·2	0·012	0·5	4·5	4·7
1 : 0	0·2	0·001	10·6	5·3	4·9
Exp. 18.					
5 : 1	0·1	0·017	0·2	4·1	4·3
15 : 1	0·1	0·006	6·7	4·5	4·5
1 : 0	0·1	0·0007	11·5	5·1	4·7
Exp. 19.					
5 : 1	0·05	0·008	4·3	4·1	4·2
15 : 1	0·05	0·003	8·6	4·4	4·3
1 : 0	0·05	0	11·8	4·9	4·4
Exp. 20.					
5 : 1	0·025	0·004	8·3	4·0	3·8
15 : 1	0·025	0·0015	8·8	4·3	4·0
1 : 0	0·025	0	11·0	4·7	4·1

Table I (*continued*).

E. *Potassium formate and formic acid.*

Exp. 21.

Ratio (Salt/Acid)	Concentration of total formate (*M*)	Concentration of CH_2O_2 (*M*)	Corrected rate of fermentation cc. per 5	p_H Beginning	p_H End
5 : 1	0·5	0·083	0·02	4·2	4·2
15 : 1	0·5	0·031	0·14	4·6	4·6
1 : 0	0·5	0·005	5·6	5·5	5·3

Exp. 22.

5 : 1	0·2	0·033	0·01	4·2	4·2
15 : 1	0·2	0·012	0·7	4·5	4·6
1 : 0	0·2	0·0009	7·5	5·3	4·9

Exp. 23.

5 : 1	0·1	0·017	0·5	4·1	4·2
15 : 1	0·1	0·006	5·0	4·5	4·5
1 : 0	0·1	0·0004	7·7	5·1	4·6

Exp. 24.

5 : 1	0·05	0·008	4·4	4·0	4·1
15 : 1	0·05	0·003	7·3	4·4	4·2
1 : 0	0·05	0	8·2	4·9	4·3

Exp. 25.

5 : 1	0·025	0·004	6·6	4·0	3·8
15 : 1	0·025	0·0015	7·5	4·3	4·0
1 : 0	0·025	0	7·9	4·6	4·1

F. *Ammonium formate and formic acid.*

Exp. 26.

Ratio (Salt/Acid)	Concentration of total formate (*M*)	Concentration of CH_2O_2 (*M*)	Corrected rate of fermentation cc. per 5′	p_H Beginning	p_H End
5 : 1	0·5	0·083	0·04	4·2	4·2
15 : 1	0·5	0·031	0·04	4·7	4·7
1 : 0	0·5	0·004	4·9	5·4	5·4

Exp. 27.

5 : 1	0·2	0·033	0·05	4·1	4·2
15 : 1	0·2	0·012	0·8	4·5	4·7
1 : 0	0·2	0·0016	7·3	5·4	4·9

Exp. 28.

5 : 1	0·1	0·017	0·4	4·1	4·2
15 : 1	0·1	0·006	4·5	4·5	4·4
1 : 0	0·1	0·0009	8·4	5·0	4·5

Exp. 29.

5 : 1	0·05	0·008	5·1	4·0	4·2
15 : 1	0·05	0·003	9·4	4·4	4·2
1 : 0	0·05	0	11·2	4·7	4·3

Exp. 30.

5 : 1	0·025	0·004	8·3	4·0	3·9
15 : 1	0·025	0·0015	10·7	4·3	4·0
1 : 0	0·025	0	11·3	4·6	4·0

p_H gradually increases as the concentration of total acetate decreases. In the 0·5 *M* curve, the point at p_H 4·7, at which the ratio of salt : acid = 2 : 1 and the amount of total acid 0·167 *M*, is a typical inflexion point, and the 0·2 *M* curve may also be regarded as having an inflexion point. These curves agree with the so-called "characteristic curve" described by Hägglund and Augustson [1925, 1]. In the 0·1 *M*, 0·05 *M* and 0·025 *M* curves, there is no inflexion point, but these curves can be considered as "characteristic curves," owing to the rapid change in rate of fermentation when the p_H value is slightly changed.

According to Hägglund and Augustson's observations [1925, 1], the optimum p_H value is variable, according to the concentration of the acetate buffer solution. The optimum value moves to the acid side in lower concentrations of the acetate buffer solution. In the present experiments an optimum p_H value was never definitely attained, although in one or two cases it was closely approached. It lies on the alkaline side of the p_H values used.

The concentration of the buffer solution appeared to have a remarkable effect on the rates of fermentation, when these were compared at constant p_H. In discussing this question, however, care should be taken to ascertain as far as possible the independent rôle of each constituent of the buffer solutions, viz. free acid, hydrogen ions, acetate ions, sodium ions, sodium acetate.

Fig. 3 shows the relation between the rate of fermentation and the amount of free acid, and it is seen that the rate is almost independent of the total concentration and of the nature of the cation. As the amount of free acid increases.

the rate of fermentation diminishes rapidly at first until the concentration of about 0·1 *M* is reached; it then falls more slowly, and gradually approaches zero. The rate, as pointed out by Hägglund and Augustson [1925, 1], is seen to be very sensitive to the presence of free acid, a concentration of 0·2 *M* reducing it from about 10–12 cc. to 0·2 cc. per 5 minutes. In short, the fact that the rate of fermentation increases as the concentration of acetate-acetic acid buffer solution decreases is principally due to the variation in the amount of free acid, as found by Johannessohn [1912], Euler and Heintze [1919].

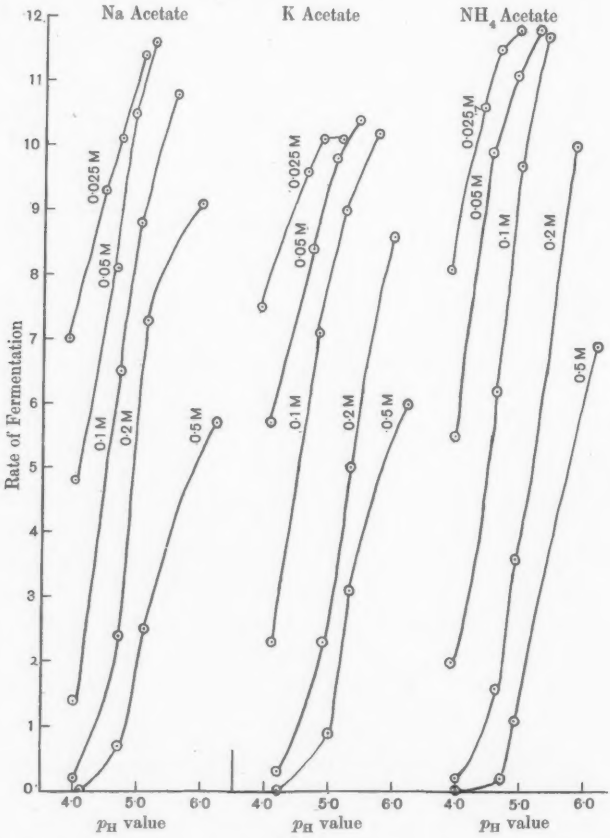

Fig. 1.

This effect is no doubt a complex one, due in part to hydrogen ions and in part to the undissociated molecules of the acid, but the influence of the latter is much greater than that of the former.

Fig. 2, which deals with fermentation in presence of formate, is of much the same character as Fig. 1. It shows that the rate of fermentation with these buffer solutions increases remarkably as their concentration diminishes, and that this does not depend upon the variety of cations ($Na^{\cdot}$, $K^{\cdot}$, and $NH_4^{\cdot}$).

Fig. 4 shows that, as in the case of the acetate-acetic acid buffer solutions, the rate of fermentation with formate-formic acid buffer solutions is controlled by the amount of free acid and is independent of the total concentration.

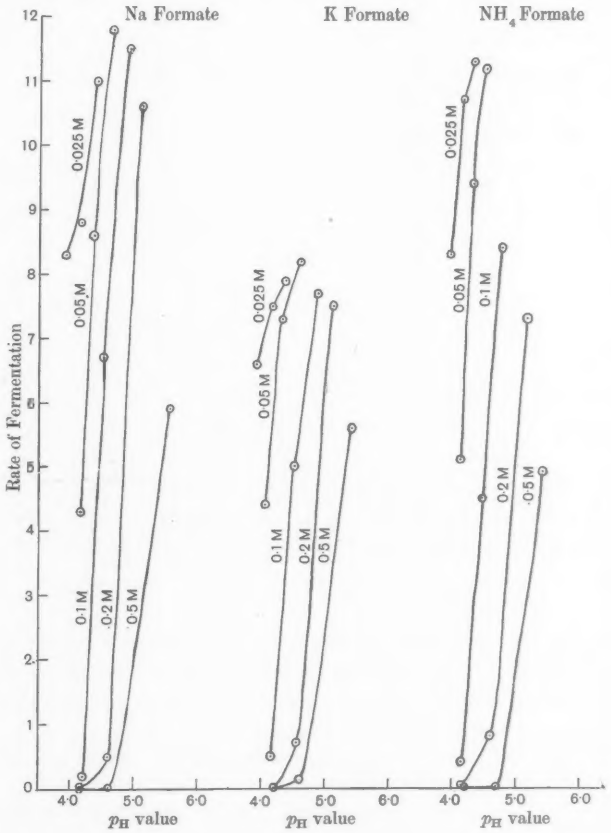

Fig. 2.

The relation between the rate of fermentation and the concentration of acetic and formic acids in the medium is approximately represented by a hyperbolic curve (see Figs. 3 and 4). The observations did not afford any evidence in confirmation of Johannessohn's observation [1912] that formic

acid and its higher homologues accelerate yeast fermentation at a sufficient degree of dilution, but the conditions of the experiment were very different from his.

No specific effect of formates, as compared with acetates, was observed. It was, however, found that the concentration of formic acid required to produce a given rate of fermentation was always much less (5–8 times) than that of the acetic acid required for the same purpose.

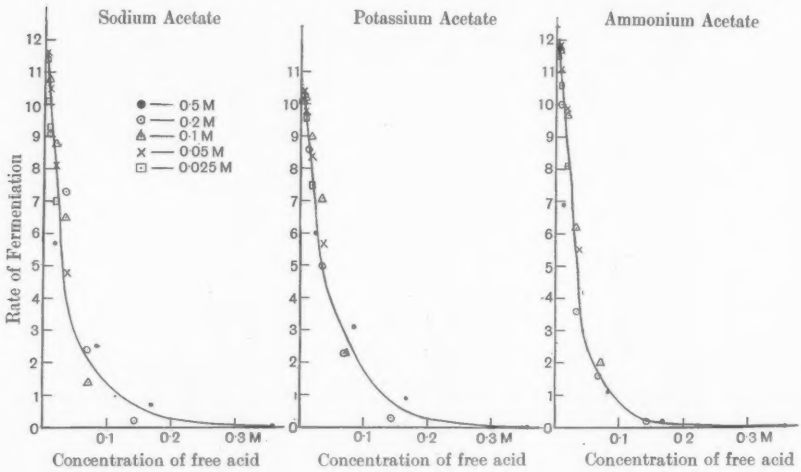

Fig. 3.

No direct comparison is possible of these results with those of Euler and Cassel [1913] and Euler [1919] in which noticeable accelerations were obtained by adding solutions of the formates of Na, K, and NH_4 to mixtures of yeast and sugar solution.

Summary.

(1) Fermentation by living yeast was observed with 0·5 *M*, 0·2 *M*, 0·1 *M*, 0·05 *M* and 0·025 *M* concentrations of acetic acid and acetate buffer solutions, the Na, K and NH_4 salts being used, and with the same concentrations of formic acid and formate buffer solutions.

(2) The rate of fermentation rapidly increased as the total amount of acid decreased, in both cases.

(3) It is suggested that a hyperbolic relation exists between the rate of fermentation and the amount of free acid in both these buffer solutions.

(4) At constant concentration of acid the rate is almost independent of the total acetate or formate concentrations.

(5) When the rate of fermentation is plotted against the p_H of the medium, the total concentration of acetate or formate (*i.e.* acid + salt) being kept constant, the form of the curve is the same for the cations, $Na^{\cdot}$, $K^{\cdot}$, and $NH_4^{\cdot}$.

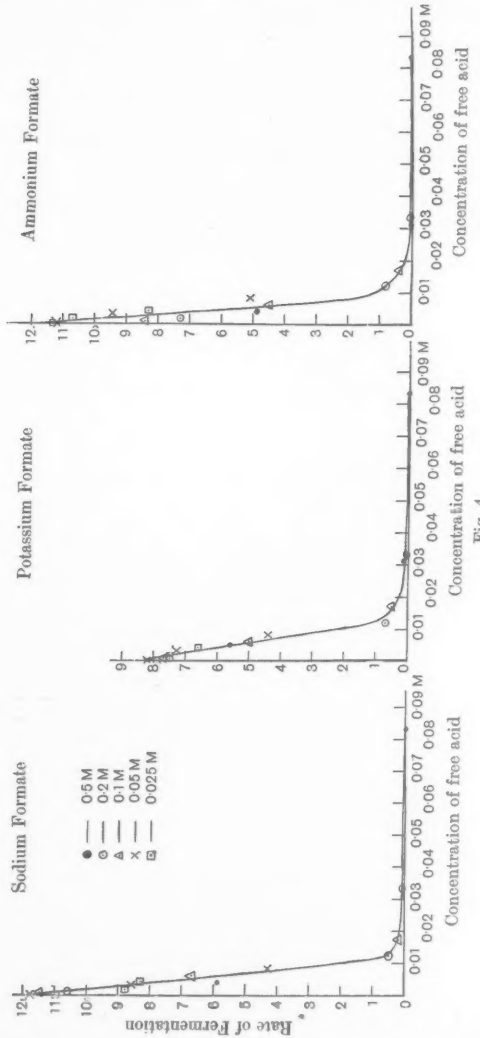

Fig. 4.

(6) No specific effect of formates as compared with acetates was observed, but formic acid is 5–8 times as potent as acetic acid in diminishing the rate of fermentation.

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LIX. STUDIES IN PURINE METABOLISM.

I. VARIATIONS IN THE NUCLEAR-PLASMIC RATIO IN THE ADULT ALBINO RAT. II. SYNTHESIS OF PURINES BY THE ADULT MAMMALIAN ORGANISM.

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I. The Nuclear-Plasmic Ratio.

One of the most fundamental problems of biology is that of the interdependence between nucleus and cytoplasm. The most striking equilibrium of the cell is that between nucleus and cytoplasm, and it is at once obvious that any factor affecting the vitality of a cell must of necessity tend also to affect the relative proportions of the above two phases. Thus the nuclear-plasmic ratio might to a certain extent be considered as an index of vitality, and as such it was put forward by Hertwig [1903], who assumed that in the case of monocytes a tendency exists towards the conservation of a constant nuclear-plasmic ratio, *i.e.* that changes in the nucleus must bring about corresponding changes in the cytoplasm, and *vice versa*. Hertwig, and the majority of subsequent workers, measured nuclear and cytoplasmic mass in terms of the areas occupied by each phase in microscopic preparations, and many interesting and far-reaching conclusions have been drawn from results so obtained. In particular, the question of the existence of protein storage has greatly occupied the attention of biologists, and it will not be out of place shortly to describe the development of research on this subject.

Rubner [1908] stated that well-nourished cells possess a reserve of proteins which may amount to about 50 % of the total cytoplasmic proteins, and which may be lost without damage to the vital functions of the cell. In the same paper he states that the dimensions of a cell are determined by its nuclear-plasmic ratio, but since the latter must necessarily be approximately doubled by the loss of half the cytoplasmic proteins, it appears difficult to reconcile this statement with that preceding. Such a condition, in which the nucleus is greatly increased relatively to the cytoplasm, has been shown by Hertwig [1904] to be fatal to *Actinosphaerium eichhornii* and other protozoa, and, as we shall later endeavour to show, should lead in these conditions (hunger) to the destruction of cells in general.

Rubner's contention is supported by the following observations. Boyd [1895], Miescher [1906] and Greene [1919] have shown that in the salmon, during the period of spawning, muscular tissue undergoes profound atrophy, in which the volume but not the number of muscle fibres diminishes. Moulton [1923] showed that cattle behave similarly in this respect, their muscle-cell volume being respectively as 14 : 8 and 6 : 1 in fat and thin and starved individuals respectively. Since in these cases in restitution the cells regain their original volumes it was assumed that the nuclei remain intact, the loss being chiefly sustained by the cytoplasm. Further observations supporting the view that in hunger the nuclear-plasmic ratio must increase are those of Morgulis [1911, 1913] on salamanders and rats, of Leonard [1887] on frogs, of Morpurgo [1888] on pigeons, and of Carlier [1892] on hedgehogs. In all these cases, with the exception of rats, the cells of the organs examined exhibited greater loss of cytoplasm than of nucleus, although these differences were not of the order indicated by Rubner.

Other workers who have on rather less definite grounds decided in favour of the existence of a protein reserve are Pflüger [1903], who held that the liver may serve as a storehouse not only of glycogen but also of proteins, Schreuer [1905], who from a study of the respiratory quotient concluded that a certain temporary and limited storage of proteins may take place in the tissues after liberal protein feeding, and Seitz [1906], who showed that the nitrogen content of the liver of starved hens and ducks is smaller in hunger than after liberal protein feeding. The evidence, however, on which the two former workers base their conclusions is both insufficient and inconclusive, whilst Seitz's results prove nothing but that the weight of the liver, and with it its nitrogen content, diminishes in hunger, and indeed, in the case of cats exactly the contrary has been stated by Schryver [1906]. For these reasons the statements of the above three workers will not here be further considered. As to the other observations cited, they cannot be considered as being so finally and decisively in favour of Rubner's theory as Robertson [1923] and Schaeffer and Le Breton [1923] contend.

In the first place, E. Godlewski jun. [1908] found that for polycellular organisms (sea-urchins) it appeared that the nuclear-plasmic ratio of the entire embryo as a whole determined its further development, and that for the study of such organisms conclusions drawn from the study of individual cells may be misleading. Further, as Robertson pointed out, the choice of organ in most of the above cases was singularly unfortunate, the liver being usually taken. As is known, liver cells serve to a certain extent as places of storage of glycogen, which may represent a considerable portion of the cytoplasm, and which would disappear at a comparatively early stage of hunger. While, therefore, it may be true that in hunger liver cells lose cytoplasm more rapidly than nucleus, it by no means follows that the same holds for other cells of the body, not possessed of a glycogen reserve.

It will also be observed that these observations are made on somewhat

heterogeneous material, the least well represented being mammals, and we may add that in the case of the rat (Morgulis) the results obtained were somewhat inconclusive, as both nucleus and cytoplasm were diminished almost equally in hunger.

Finally, both nuclear and cytoplasmic material is to a certain extent diluted in hunger, and we have no reason *a priori* to assume that this dilution is equal for both constituents. This objection renders extremely questionable the value of observations based on morphological methods, and indicates the necessity of some gravimetric procedure.

The first research in this direction is that of Masing [1910], who took as representative of the cytoplasm, protein, *i.e.* total nitrogen, and nucleic acid phosphorus as characteristic of the nucleus. That nucleic acid is a true measure of nuclear mass has been shown by the experiments of Kossel [1886], Tichomiroff [1885], Burian and Schur [1897], and Fridericia [1912]. Schaeffer determined nucleic acid as purine nitrogen, and since this method presents certain advantages to that of Masing it has been used in this research.

Purine storage. A second problem connected with the nuclear-plasmic ratio is that of the possibility of storage of nuclear material. Should this exist, feeding with foods of high purine content should lead to an increase in the nuclear-plasmic ratio. Numerous researches on purine metabolism have yielded a mass of confused and often conflicting evidence as to the fate of ingested purine bodies. Thus it is generally known that in man uric acid excretion falls to a minimum on a purine-free diet, that only a portion of the ingested purines is excreted as uric acid, the remainder being destroyed in the intestine by bacterial action, and that protein catabolism is accelerated by purines, as also the reverse [Underhill and Farrell, 1922]. Finally, it is known that injection or ingestion of purines leads to a considerable increase in the number of leucocytes, which on destruction yield a further quota of uric acid.

From this evidence it is difficult to arrive at any conclusion as to the possibility of a purine reserve, and, in order to decide this question, a series of rats, previously kept for some months on a purine-free diet, have now been given purine-containing foodstuffs, and their nuclear-plasmic ratio compared with that of control animals.

Experimental.

Albino rats were used, as they had the advantage of being able rapidly to degrade extracellular purines to allantoin, so that the values obtained for purine nitrogen represent almost exclusively those purines which are actually in the nucleus. These rats were fed for several weeks with purine-free foods, *i.e.* bread and milk. They were killed by strangulation in order to avoid loss of blood or introduction of foreign substances, and were immediately weighed, the contents of the alimentary tract removed, and again weighed. They were

then minced, the mince well mixed, and two portions of 50 g. and 20 g. weighed out, serving for hydrolysis and determination of percentage solid substance respectively. The first portion was hydrolysed by boiling for 4 hours with 500 cc. 4 % sulphuric acid, and from the hydrolysis mixture two samples were taken for the determination of total nitrogen by Kjeldahl's method. This procedure is necessarily somewhat crude, but it is difficult to conceive of any better method of obtaining a representative sample in small enough quantity for this purpose. In certain cases, however, the following procedure was adopted as a check, in addition to that preceding. A weighed portion of mince was moistened with dilute hydrochloric acid, in order to prevent loss of ammonia, and dried to constant weight at 100°. The dried mass, together with the vessel, was then again weighed, the residue powdered in a mortar, and aliquot portions taken for analysis. The values so obtained differed very slightly from those obtained from the hydrolysate.

The purine-nitrogen was in all cases determined by the method of Krüger and Schittenhelm [1905], in which the purines are doubly precipitated as the copper salt, the nitrogen being then determined by the Kjeldahl method.

The proportion of solid substance was determined by drying to constant weight at 100°. Under these conditions not all the water was removed, since at 110° a further small decrease in weight could be obtained. However, the difference was not great, and for comparative purposes drying at 100° was quite adequate, and for technical reasons preferable.

The calculations are as follows. The total volume of the hydrolysate at room temperature was 550 cc., and from this two portions of 20 cc. were taken. Each portion represented 2/55 of 50 g. of mince, and the percentage of nitrogen was therefore obtained by multiplying the nitrogen content of each sample by 55. The value obtained for purine-nitrogen was corrected by multiplying by 55/51, and doubled, to give percentage. As to the nuclear-plasmic ratio, this is not given exactly by the division of purine by total nitrogen, since the latter includes also the former. Schaeffer expresses this ratio as $N_P/N_T\text{-}N_P$, but, as he points out, this also is not exact, since nucleic acid contains also pyrimidine-nitrogen, and, further, certain nitrogenous substances other than nucleic acid may be present in the nucleus, so that the absolute values would undoubtedly be higher than those calculated on the above basis.

For comparative purposes, however, the form in which the nuclear-plasmic ratio is expressed is not of essential importance, and, in this case, that used by Schaeffer has been adopted.

Control animals.

The values obtained for control animals are given in Table I. As can be seen, the sex of the animals does not influence the results, and, whilst wide variations exist in the percentage of solid substance, from 30·4 to 37·5 %, in the nitrogen content from 3·0838 to 3·5944 % live weight and from 8·253 to 11·600 % dry weight, and in the purine nitrogen content from 59·0 to

72·8 mg. % live weight, and from 168·5 to 224·0 mg. % dry weight, yet the nuclear-plasmic ratio is fairly constant, varying from 18·30 to 22·34 $\times 10^{-3}$, with a mean value of 20·62 $\times 10^{-3}$, and this is taken as the normal figure.

Table I. *Control Animals.*

No.	Weight without contents of alimentary canal, g.	% solid substance	% N live weight	% N dry weight	Purine-N, mg. % live weight	Purine-N, mg. % dry weight	Nuclear plasmic ratio
1 ♂	166·4	30·4	—	—	62·1	204·3	—
2 ♂	161·2	31·9	—	—	61·3	192·0	—
3 ♂	172·0	34·5	—	—	65·0	189·45	—
4 ♂	172·4	31·0	3·3370	10·7655	67·8	218·7	20·74 $\times 10^{-3}$
5 ♀	154·4	37·5	3·0958	8·253	62·3	166·1	20·52 × „
6 ♂	160·5	35·05	3·3310	9·501	72·8	207·6	22·34 × „
7 ♂	167·7	33·5	3·3018	9·856	—	—	—
8 ♀	135·7	35·05	3·3048	9·428	59·0	168·5	18·20 × „
9 ♂	124·0	31·0	3·5944	11·600	69·4	224·0	19·70 × „
10 ♀	147·3	33·0	3·0838	9·346	65·8	199·5	21·81 × „
11 ♀	135·4	35·05	3·2278	9·208	66·4	189·5	21·01 × „
	Mean	33·45	3·2846	9·745	65·2	196·0	20·62 × „

Starvation.

Table II gives the values obtained for rats subjected to inanition. These rats lived for from 74 to 216 hours, this depending upon the room temperature and the initial state of nourishment, and lost from 20 to 40 % of their original weight. Here the percentage of solid substance varies from 28·9 to 31·6, on the average 30·45, pointing to a certain dilution of the tissues. The total nitrogen, which varies from 3·2796 to 4·1195 and 10·840 to 13·764 % live and dry weights respectively, has the average values of 3·7185 and 12·215—the latter being almost 25 % above the normal value. Purine-nitrogen is, on the whole, lower, being from 54·55 to 72·16 mg. % live weight, but calculated on dry substance is also higher than normal, namely from 176·0 to 232·8, on the average, 207·7 mg. %. The effect of this increase in total nitrogen, together with a slight decrease in purine-nitrogen, is to lower the nuclear-plasmic ratio to a mean value of 17·77 $\times 10^{-3}$, varying from 16·35 to 21·41 $\times 10^{-3}$. This mean value is 15 % below normal, and as, according to Schaeffer, the margin of experimental error is $\pm$ 5 %, this difference must be considered as being substantiated.

Table II. *Inanition.*

No.	Hours hunger	Initial weight g.	Final weight g.	% loss	% solid substance	% N live weight	% N dry weight	Purine-N mg. % live weight	Purine-N mg. % dry weight	Nuclear-plasmic ratio
1 ♂	89·0	155·5	116·4	29·97	31·0	3·3774	10·895	54·55	176·0	16·42 $\times 10^{-3}$
2 ♂	74·5	138·0	109·0	20·29	29·8	3·3726	11·318	57·46	192·9	17·33 × „
3 ♂	78·0	202·5	159·4	21·28	28·88	3·2796	11·360	—	—	—
4 ♂	134·0	159·2	106·0	33·41	30·5	3·8494	12·621	63·88	209·4	16·88 × „
5 ♂	139·0	119·3	79·0	33·72	31·11	3·9501	12·697	—	—	—
6 ♂	96·0	177·5	118·8	33·06	31·0	3·6844	11·898	72·16	232·8	21·41 × „
7 ♀	223·0	182·9	114·7	37·27	30·25	4·0664	13·443	—	—	—
8 ♀	216·0	173·7	103·1	40·67	29·9	4·1195	13·764	66·28	221·7	16·35 × „
9 ♀	186·5	145·9	101·1	30·68	31·6	3·4265	10·840	65·26	206·5	19·42 × „
10 ♀	186·0	179·7	114·5	35·45	30·5	4·0594	13·310	65·52	214·8	16·40 × „
Mean	—	—	—	—	30·45	3·7185	12·215	63·6	207·7	17·77 × „

Purine feeding.

The results of purine feeding are found in Table III. These rats were fed for from 6 to 14 days with bread and milk to which nucleic acid had been added, then for three days without purines to clear the body of unassimilated purines which might transitorily remain, and then killed.

The values obtained do not differ greatly from the normal, the nitrogen content being on the average 3·410 and 10·281 % live and dry weights respectively, and varying from 3·057 to 3·931 and 9·306 to 11·911 %. This slight increase in total nitrogen is accompanied by a similar slight decrease in purine-nitrogen, which is 63·56 and 191·9, varying from 59·64 to 70·74 and 170·1 to 214·3 mg. % live and dry weight. The nuclear-plasmic ratio is thus lowered to $18{\cdot}85 \times 10^{-3}$, varying from 18·32 to 20·97 $\times 10^{-3}$.

Table III. *Purine feeding.*

No.	Period of purine feeding, days	Weight g.	% solid substance	% N live weight	% N dry weight	Purine-N mg. % live wt.	Purine-N mg. % dry wt.	Nuclear-plasmic ratio
1 ♂	19	214·0	35·05	3·2617	9·306	59·64	170·1	$18{\cdot}62 \times 10^{-3}$
2 ♂	21	195·9	34·5	3·3349	9·665	61·51	178·3	18·79 × „
3 ♂	24	185·8	31·0	3·0570	9·860	62·78	202·5	20·97 × „
4 ♀	6	147·0	32·5	3·4650	10·663	63·12	194·2	18·55 × „
5 ♂	8	191·9	33·0	3·9308	11·911	70·74	214·3	18·32 × „
Mean	—	—	33·1	3·410	10·281	63·56	191·9	18·85 × „

Table IV. *Synthesis.*

No.	Period of purine feeding, days	Weight g.	% solid substance	% N live weight	% N dry weight	Purine-N mg. % live wt.	Purine-N mg. % dry wt.	Nuclear-plasmic ratio
1 ♂	—	164·9	32·0	3·2017	10·006	70·2	218·9	$22{\cdot}42 \times 10^{-3}$
2 ♂	—	138·9	36·8	3·2047	8·708	64·9	176·4	20·63 × „
3 ♂	—	131·1	30·5	3·2917	10·790	73·52	241·1	22·84 × „

II. Synthesis of Purines.

The question of whether the adult mammalian organism is capable of synthesising purines, or whether these have to be supplied with the food, has for many years been debated, and in spite of numerous researches, carried out mainly on man, no definite conclusion can so far be drawn. These researches consisted mainly in a comparison of the amounts of administered and excreted purines over a given period of time, and on such grounds Schreiber and Waldvogel [1899], Burian and Schur [1900], Fauvel [1906], Umber [1909], Benedict [1916], and Kollman [1921] have decided in favour of purine synthesis. Owing, however, to the difficulty of supplying food of constant and known purine content over a long period of time, and to the impossibility of the quantitative determination of excreted purines, these results cannot be considered as being entirely conclusive. In this group, the

best substantiated work is perhaps that of Benedict, who kept a Dalmatian dog for over a year on a so-called purine-free diet, and found that over this period 100 g. of uric acid had been excreted.

On the other hand, Abderhalden [1914], Fürth [1915] and Hammarsten [1914] consider that the synthesis of purines by adult mammals cannot be considered as proved. Since, therefore, this might still be regarded as an open question, restitution experiments have been carried out in the present research, consisting in the partial inanition of a number of rats, which were then fed until sufficient restitution of weight had taken place, when they were killed and their purine content determined. One of the chief advantages of this procedure is that the period over which experiments are conducted is extremely short in comparison to that necessary for metabolism experiments in which a purine balance is drawn up, and which necessarily involve the introduction of larger quantities of foodstuffs whose purine content it is difficult to determine with any degree of accuracy.

Experimental.

A number of rats were kept without food until their weight had fallen to about 80 % of the original value. They were then fed with a diet adequate in all respects, consisting of sugar, starch, caseinogen, Witte's peptone, purified lard, cellulose, and mineral salts (Osborne and Mendel's formula) together with the necessary vitamins, which were supplied either by a known small quantity of milk, or by lemon juice.

On this diet restitution took place fairly rapidly, and, when the weight had sufficiently exceeded that at the end of the period of hunger, the animals were killed and their purine content determined.

Table IV shows that the total and purine-nitrogen contents are similar to those of Table I, as is the nuclear-plasmic ratio. Table V gives the purine-nitrogen contents of the same animals at the beginning of the experiment and at the end of the hunger period, calculated from the average values found in Tables I and II. In calculating these values, the average weight of the contents of the alimentary canal, namely 10 g. for control and 4 g. for starved animals, was subtracted from the weight, and the result multiplied by 0·65 and 0·636 respectively. The final purine-nitrogen content was obtained by direct determination, and by subtracting from this the hunger value we have the gain in purines over the period of feeding.

Table V. *Synthesis of purines.*

No.	Original weight	Weight after hunger	% loss	Weight after restitution	Original N_P (65 mg. %)	Hunger N_P (63·6 mg. %)	Restitution N_P	N_P/gain during feeding	N_P given in food	N_P synthesised	% gain	Hours feeding
1 ♂	187·0	138·6	26·2	164·9	121·7	88·16	115·7	27·5	3	24·5	27·8	341
2 ♂	162·0	106·0	34·57	138·9	105·3	67·4	90·2	22·8	3	19·8	29·38	486
3 ♂	141·0	110·0	22·0	131·1	91·6	70·0	96·4	26·4	0	26·4	37·7	360

The first rat weighed at the end of the period of starvation 138·6 g., and was killed after feeding for 340 hours, in which period 400 cc. of milk were consumed, the gain in weight being 26 g. During this time 27·5 mg. of purine-nitrogen had been gained, and if from this we subtract 3 mg. of purine-nitrogen contained in the milk this leaves 24·5 mg. purine-nitrogen synthesised, or an increase of 27·8 % of the purine content at the end of the period of hunger. Similarly, the second rat after starvation weighed 106 g., and after feeding for 486 hours, 400 cc. of milk being also consumed in this time, 33 g. more. Here the gain is 22·8 mg., and the amount synthesised, after subtracting 3 mg. for the milk, is 19·8 mg., an increase of 29·4 %. The third rat received the same diet, with the difference that in place of milk, lard containing a certain amount of vitamin A was given, and in this case the purine content of the food was entirely negligible. The period of feeding was in this case 360 hours, during which time the weight rose from 110 to 131 g. Here the amount of purine-nitrogen synthesised was 26·4 mg., representing an increase of 37·7 %.

Discussion.

Inanition. In view of the results shown in Table II it appears that Rubner's theory of protein storage cannot be considered to be substantiated, at least so far as mammals are concerned. On the contrary, it appears that in hunger rats exhibit a greater loss of nucleus than cytoplasm. It becomes therefore necessary critically to consider the theories and experimental findings of Rubner and of other workers in this field, and to attempt to reconcile these data with our own findings.

If one accepts that in hunger the cytoplasm is used up more rapidly than the nucleus, a time must arrive when the cell can no longer remain in equilibrium with the latter.

It is well known that the nucleus is the seat of perhaps the most important reactions of life, and that the cytoplasm must supply the energy demands of its nucleus. In this connection it is not without interest to cite certain experiments of Gerassimow [1902] on infusoria, in which the nuclear-plasmic ratio of those cultivated at 8° was 1/7, whilst at 25° it fell to 1/13. This points to the retardation of chemical processes at lower temperatures, necessitating a nucleus almost twice as great as that which suffices at 25°, and might to a certain extent explain the low energy loss of poikilothermic animals at lower temperatures, since their nuclei become then insufficient to carry out all the reactions necessary to normal life.

Robertson considers that a cell, which, as a result of starvation, has suffered disproportionate loss of cytoplasm, is rejuvenated, and should tend to undergo mitosis. It is difficult to conceive in what way this could lead to the restoration of the equilibrium of the cell, since the nuclear-plasmic ratio remains constant with each division, and, on the contrary, since the nuclear surface increases relatively to its mass, each mitosis should intensify the pathological condition of the cell.

Since, in our case, cytoplasmic material is not available, the cell can regain equilibrium only by the destruction or extrusion of nuclear material. Should it be unable to do this, the cell must break up as a whole and, in that case, its cytoplasmic constituents may be utilised by other cells, whilst the nuclear material is in all probability rejected. Such a condition would necessarily lead to a diminution of the nuclear-plasmic ratio for the entire organism.

In support of the above reasoning may be cited certain observations of Hertwig [1904] and of Popoff [1908, 1909] in which it is shown that in infusoria nuclear hypertrophy leads either to the degeneration of part of the nucleus to chromidium (unorganised nuclear matter) which is distributed throughout the cytoplasm, or to the death of the organism as a result of the extrusion of the entire nucleus. Further, in so-called depressed infusorial cultures, in which multiplication has ceased, the organisms exhibit nuclear hypertrophy, so that, at least in this case, increase in the nuclear-plasmic ratio does not lead to mitosis, but, on the contrary, inhibits it. Popoff was also able to show that in infusoria the removal of a portion of cytoplasm immediately before mitosis considerably retarded the latter.

Finally, it is in this connection interesting that in man the uric acid content of the blood rises during starvation, particularly during the pre-mortal period [Lennox, 1924]. This is obviously due to augmented nuclear destruction, and it appears that in rats a similar process takes place, with the difference that uric acid is in this case degraded to allantoin.

Purine feeding.

The nuclear-plasmic ratio falls as a result of purine feeding to $18{\cdot}85 \times 10^{-3}$, or 9 % below normal. This result excludes the possibility of any storage of purines taking place in the rat, and further suggests that introduced purines are not anabolised by the mammalian organism.

As to the reason for an actual relative reduction of purines, it would appear either that these have an accelerating effect upon cellular, particularly nuclear, metabolism or that the leucocytosis observed by Schittenhelm after the ingestion of purines leads to enhanced excretion of endogenous uric acid or allantoin, and so diminishes the purine content of the entire organism.

SUMMARY.

1. The nuclear-plasmic ratio has been determined for albino rats under various conditions.

2. In hunger the nuclear-plasmic ratio falls slightly below normal, indicating a greater relative loss of nuclear than of cytoplasmic material.

3. The protein reserve theory postulated by Rubner must in view of this be reconsidered.

4. Purine feeding does not lead to any increase in the nuclear-plasmic ratio, whence it follows that purine bodies are not assimilated and stored by the mammalian organism.

5. Purine bodies are synthesised with comparative facility by the adult albino rat.

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